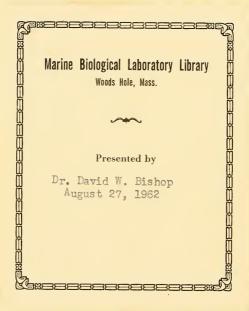# Spermatozoan
Motility

Spermatozoan Motility

A symposium organized by the American
Society of Zoologists, cosponsored by the
Society of General Physiologists, and pre-
sented at the New York meeting of the
American Association for the Advancement
of Science, 29–30 December 1960

Edited by DAVID W. BISHOP

Preface

With the ever increasing tendency of scientists of different disciplines to integrate and join forces in a concerted attack on a central biological problem, it is perhaps not surprising to find on one podium three cytologists, three biophysicists, four biochemists, two physiologists, two practitioners, and two, if indeed not many, philosophers. Nor is it surprising that having once convened, such a meeting of minds might produce a highly fruitful, sometimes spirited, and generally provocative exchange of view. That men from two hemispheres would gather, during the Christmas holiday no less, to discuss the characteristics, significance, and mechanism of spermatozoan motility is perhaps less to be expected. Nevertheless, it was with such anticipation in mind that this symposium was conceived and organized, and this volume attests to the success of the venture.

The problem of sperm motility is literally as old as the microscope itself. Its fascination is apparent to every student of zoology and its phylogenetic ubiquity a signal reminder that Nature is not inclined to change the model merely to be fashionable. The mechanism of sperm movement has long been a perplexing problem, whether viewed as a complex machine dependent on chemomechanical coupling processes or as an undulating micro-organelle in hydrodynamic equilibrium with its environment. The ultimate mechanism of sperm flagellation, a central theme of this conference, would seem to bear certain overtones in relation to motile processes in other types of cells, including free-living flagellates, ciliary systems, and muscular tissue as well. The symposium has brought out a number of new facts and some older interrelations bearing on these and similar problems Speculation was frequent and was encouraged; no other area can more appropriately lay claim to Bertrand's timely remark, "Give me three constants and I will draw an elephant; give me four and I will make him wave his trunk."

Some liberties have been taken in the arrangement of material for publication. The comments of the several able chairmen of the ses-

sions, H. B. Steinbach, R. J. Flipse, and T. Hayashi, have been included as contributions. An additional paper (R. Rikmenspoel) was added as a result of highly appropriate comments from the floor. We are indebted to Sir James Gray, unfortunately unable to attend, for his willingness nevertheless to contribute an introductory chapter.

The symposium, made possible by a generous grant from the U.S. Public Health Service through the National Institutes of Health, was held under the joint aegis of the American Association for the Advancement of Science and the American Society of Zoologists, and cosponsored by the Society of General Physiologists. To all who contributed to the presentation and publication of this symposium sincere gratitude is due.

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Contents

Introduction

Flagellar Propulsion

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Not so many years ago an interest in the mechanism of flagellar movement was restricted to a very small group of biological specialists. Today it is attracting almost as wide a range of high-powered scientific effort as any other biological problem; mathematicians, physicists, chemists, and biologists all find common ground. And so, if some omniscient body were to tell me exactly how such systems work, I would feel very embarrassed; the answer would involve a knowledge of hydrodynamics and biochemistry far beyond my comprehension. But, from time to time is is useful to take stock of what we know and consider how to collect more information. The proceedings of this symposium fulfill this need and provide the stimulus for further effort.

If we visualize the tail of a spermatozoon as a piece of aquatic propulsive machinery, three questions at once arise. (1) What are the composition, shape, and mutual relationship of each part of the prime mover or engine? (2) What is the nature of the fuel, and how is its chemical energy made available for useful work? (3) What are the precise shape, path of motion and frequency of movement of the propeller surface? One cannot approach any of these questions without realizing how far we have yet to go to find a comprehensive answer, but at the same time feeling that we are steadily, if slowly, moving in the right direction. The following notes deal almost solely with the third and simplest of these three questions.

THEORY OF FLAGELLAR PROPULSION

The propulsion of nearly all living organisms depends on the principle that periodic changes in the form of the body take place against a

1

suitable external resistance. In a flagellum the changes in shape are effected by bending each short length of the filament, and the external resistance is provided by the viscosity of the medium. The waves of changing curvature passing distally along the filament are an expression of the fact that each short element is not only bending but also executing transverse movements in a plane normal to the axis of vision and that the phase of bending reached by any element at any instant is slightly ahead of that of its next posteriorly situated neighbor. The form of the waves depends on the radius of curvature exhibited by the elements when bent to their maximum extent and on the difference of curvature exhibited by adjacent elements at any particular moment. In order to elicit a forward thrust from the medium, an element must move with its surface inclined at an angle (θ) to its transverse path of motion; in other words, as it sweeps from side to side, it must have a component of motion normal to, as well as one tangential to its surface (Gray and Hancock, 1955). Under such conditions a forward thrust ensues provided the coefficient of resistance, C_D, to movement in a direction normal to the surface is greater than that, C_L, to movement tangential to the surface; Hancock (1953) has shown that for very thin and slowly moving filaments C_N equals $2C_L$. Thus the propulsive force, dF, exerted by an element at any instant is determined by three factors: (1) the tangential drag coefficient, C_L; (2) the element's velocity of transverse displacement, dy/dt; and (3) the angle between its surface and its path of motion, dy/dx.

$$dF = C_L \frac{dy}{dt} \frac{dy}{dx}$$

If we are prepared to assume that the form of the waves is the same as that of a sine curve of appropriate length, λ, and amplitude, b, it is possible to express the speed of propulsion ($\overline{V}_z$) of the filament in terms of the form and frequency, f, of the waves passing distally along it (Taylor, 1951, 1952; Hancock, 1953). This relationship is independent of the absolute value of C_L.

$$\overline{V}_z = \frac{2f\pi^2 b^2}{\lambda} \left(\frac{1}{1 + \frac{4\pi^2 b^2}{\lambda^2}} \right)$$

After allowing for a reduction in speed caused by the drag of an

inert head, the calculated and observed speeds of progression of the sperm of a sea urchin (*Psammechinus*) were found to be almost identical (Gray and Hancock, 1955).

So far, so good; but it is necessary to look rather carefully at the assumptions which underlie the theory and decide how far they can be justified by observation. These assumptions are at least four in number. (1) The transverse movement is identically the same for all elements; in other words, the wave does not change its wavelength or amplitude as it moves along. (2) All elements execute their movements in one and the same plane; there must be no tendency for the filament to rotate about the axis of the wave. (3) All transverse forces acting on the filament in directions normal to the axis of progression summate to zero throughout each complete cycle; in other words, the wave is symmetrical about its longitudinal axis, and there is no tendency for the filament to yaw in the plane of motion of its constituent elements. (4) The length of the filament is much greater than the length of a single wave and is such as to constitute an integral number of waves. So far as is known, no living system conforms strictly with these requirements. All we can safely say in the case of *Psammechinus* sperm is either that discrepancies due to each of these factors are relatively small or that collectively they cancel each other out.

OBSERVATIONAL DATA

If the transverse displacements of all elements of a flagellum were identically the same and occurred in one and the same plane, the "envelope" seen under dark-ground illumination would be that of a rectangular lamina when viewed in a direction normal to the plane of this movement. In practice the transverse amplitude of elements near the head of the sperm is always less than those near the tip of the tail (Gray, 1955, 1958). Further, a tendency to roll is one of the most characteristic features of an active spermatozoon, and this is, in itself, very strong presumptive evidence that all transverse movements are not taking place in one and the same plane. If for any reason a spermatozoon of a sea urchin is prevented from rolling, it characteristically swims along a curved path (Gray, 1955); a similar phenomenon has recently been reported by Blokhuis (1961) in bull sperm. The ability of such systems to progress along a straight

axis depends on the fact that the sperm is both yawing and rolling as it moves forward (Gray, 1955). There are thus very substantial differences between the observed movements and those which form the basis of a calculated speed of progression.

THREE-DIMENSIONAL WAVES

From time to time—indeed, during this symposium—the concept of three-dimensional waves has been invoked to account for the rotation of a sperm about the longitudinal axis of its progression. To this there can be no objection provided there is no ambiguity about what is meant by a "three-dimensional" wave. It is convenient to start an analysis of such movements from the assumption that all the elements of a filament are bending simultaneously in two internal planes normal to each other, e.g., dorsoventral and lateral. If two conditions are satisfied—and only under such conditions—a filament generating the waves could be wrapped round the surface of a circular cylinder during all phases of its activity; these conditions are (1) that the maximum degree of bending should be the same in both planes and (2) that the phase of bending reached by an element in one plane is always one-quarter of a cycle behind that reached by it in the other. When generating waves of this particular type the envelope of the filament would appear as a circle when viewed along the axis of the circumscribed cylinder, and every element would move round this circular orbit once during the passage of each complete wave. If such terms as "three-dimensional" or "helical" are restricted to waves of this particular form, misunderstanding is likely to occur. The elements constituting such waves cannot perform their circular motions against the resistance of the water without generating couples tending to rotate the whole filament about the axis of the wave in a direction opposite to that in which the elements are traveling in their circular orbits (Fig. 1) (Taylor, 1952; Gray, 1953). The net result of rolling the filament about the axis of the wave is to reduce the speed at which each element is traveling along its own orbit and to reduce the speed at which the whole wave is traveling backwards relative to fixed axes and to the surrounding water (Fig. 2) (Gray, 1953). In the limiting case when the filament rolls through 360° with the same frequency as that at which the waves are travel-
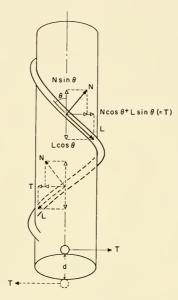
Fig. 1. In a regular helical system all the forces (normal and tangential) exerted on a wave act tangentially to the surface of a cylinder. For any one element the normal force N has a forward component $N \sin \theta$ and a transverse component $N \cos \theta$; the tangential drag L has a backward component $L \cos \theta$ and a transverse component $L \sin \theta$. For an element situated one-half of a wavelength from the first, the total transverse force will, with that of the first element, yield a couple turning the elements and the body as a whole about the axis of the cylinder in a direction opposite to that in which the elements are moving round the cylinder. During steady motion $N \sin \theta = L \cos \theta$, while the "spinning" couple is $d \cdot L \operatorname{cosec} \theta$, where d is the diameter of the helix.

ing over the filament, there is no translatory displacement of any part of the filament relative to the water; each element is then simply spinning about its own longitudinal axis; there is no movement of the wave relative to the ground and no propulsion can occur. If, therefore, a spermatozoon rolls with the same frequency as it gen-
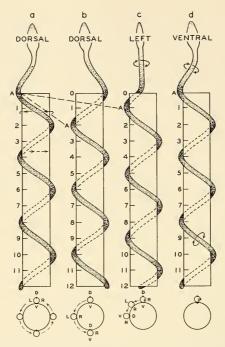
Fig. 2. Diagram illustrating the effect of axial spin on the movement of helical waves. The movement of the waves is indicated relative to a smooth cylinder which maintains a constant distance behind the animal's head, which is flattened dorsoventrally. Dotted areas show regions of the body lying over the cylinder. The wavelength is four units.

In Fig. 2a, the organism is at rest and presents its dorsal surface to the observer; the most anterior wave crest A is at zero on the cylinder. If each element of the body executes one-half of a complete contractile cycle while the body is completely restrained from spinning, each wave crest travels one-half wavelength backwards along the cylinder (Fig. 2b); if the organism as shown in Fig. 2a spins through 90° whilst undergoing one-half of a contractile cycle, the wave crests travel only one-quarter of a wavelength as in Fig. 2c; if the body spins 180° during each half-contractile cycle, the wave crests remain stationary as in Fig. 2d.

Note that for any given element the phase of contraction is exactly the same in Fig. 2b–d, viz., half-cycle ahead of that in Fig. 2a; in Fig. 2b, the organism still presents its dorsal surface to the observer at the end of the half-cycle contraction, whereas it presents its left surface in Fig. 2c, and its ventral surface in Fig. 2d. In each case the direction of "spin" is opposite to that in which each element of the system moves round the circumscribed cylinder.

6

erates waves, the form of the latter cannot be such as allow the fila-
ment to be wrapped over the surface of a circular cylinder.*

On the other hand, if the orbit of rotation of each element about
the axis of the wave is elliptical instead of circular, forward pro-
pulsion can occur even if the frequency of roll is the same as that of
the waves. An elliptical orbit could be produced in at least two ways:
(1) by a difference in the maximum degree of bending of an ele-
ment in the dorsoventral and lateral planes while maintaining a
phase difference of one-quarter of a cycle between the two cycles, or
(2) by an alteration in the phase difference of the cycles but keeping
the maximum degree of bending the same in the two planes. With
a phase difference of less than a quarter cycle, the orbit of each ele-
ment about the axis of the wave is elliptical. In the extreme case
when the two bending movements are either in phase or 180° out
of phase with each other, the path of movement of each element is
a straight line inclined at an angle of 45° to its dorsoventral axis;
the wave then becomes "two-dimensional." Risk of ambiguity would
be reduced if less emphasis could be placed on the "dimensions" of
the waves and more on the movements of constituent elements
relative to the longitudinal axis of the system.

The planes of transverse movement of the elements of the tail of
a spermatozoon could be determined if it were possible to observe
the form of its envelope when viewed along the longitudinal axis
under conditions which effectively prevent rolling of the whole fila-
ment. If rolling occurs, an elliptical orbit is transformed into a com-
plicated curve comparable with those figured by Perrin (1906) in
Cristaspira when in active undulatory movement. Much valuable
information could perhaps be obtained by photographing a sper-
matozoon in the normal way using very short electronic exposures
and lenses of very small depth of focus, thus detecting and measur-
ing any changes in the plane of an element's transverse movement
during each complete cycle.

It is generally assumed that the movement of the elements of a
flagellum relative to each other is entirely due to bending deforma-
tion and that one element cannot be displaced relative to its neigh-

* If the base of a flagellum is fixed to a cell in an epithelium (as in the choano-
cytes of a sponge), individual elements are effectively prevented from spinning
about their own longitudinal axes; they can then move in circular orbits and yet
provide an efficient means of creating a current of water.

bor by torsional rotation. This may not be true. If the bending of each element occurs in the same plane throughout its whole cycle, while successive elements are twisted relative to their neighbors, the envelope of the whole filament would form part of a screw surface and the system would roll as it moved forward (see Gray, 1955). The concept of a filament actively propagating torsional waves by internal effort may be somewhat hypothetical, but it is relevant to a question asked by Rothschild (1961) "Can a spermatozoon roll about its longitudinal axis when its tail exhibits no bending waves?" A cat dropped upside down rights itself in the air by appropriate torsional movement of different parts of its body. It would be interesting if the physicists could tell us whether a similar acrobatic feat could be performed in systems with negligible inertia.

Perhaps the moral to be drawn from all this is the need for more precise information about the actual changes in form executed by a moving flagellum by simultaneous microscopic observation in more than one plane. Until such a technique is available, it is unwise to overstress the significance of theoretical estimates of propulsive speed which are based on relatively simplified systems. It would be most valuable if mathematicians could extend their analysis to (1) waves of changing amplitude and length, and (2) systems in which the path of displacement of each element is elliptical and the whole system rolls about its longitudinal axis at a known frequency. They might well say, "Yes, if biologists will give us adequate quantitative data on which to base the calculations."

MECHANISM OF BENDING

When subjected to two equal but opposite bending couples, an elastic rod bends in the plane of the applied couples; one side of the rod is stretched and the other is compressed, energy being stored in both regions. In a flagellum the couples are generated by internal effort, but neither the structures concerned with the development of tension or those which resist compression can be identified with any certainty. On the analogy of muscle, it seems reasonable to regard the radially arranged ultramicroscopic fibrils as actively contractile, relegating compression to the action of hydrostatic pressure or to structures thus far unidentified. The situation would be clearer if it were possible to isolate a single fiber and see whether it changed

its length when treated with adenosine triphosphate (ATP) while remembering that the total degree of shortening involved by bending a very thin filament may be very small.

That a radial arrangement of contractile elements should produce bending in one plane is not restricted to flagella or cilia; it occurs in a large number of other undulating organisms and involves no great mechanical difficulty provided there is a suitable difference in phase between each of the contractile elements (Bradfield, 1953; Gray, 1955). All elements could execute the same cycle of changes in length and tension and each contribute to the energy required for uniplanar bending (Gray, 1955, p. 798). But why there should always be nine radial elements remains a mystery.

A more amenable problem concerns the mechanism which controls the phase difference between adjacent regions of the filament. How far is this maintained by an intrinsic timing mechanism and how far by forces exerted against the filament by the external medium? Or as Brokaw (this symposium) has put it, how far is the effort of contractile units exercised against internal as opposed to external resistances? The frequency of the waves is undoubtedly affected by changes in the viscosity and other physical properties of the medium, but until we know the precise changes, if any, which such factors exercise on the amplitude and length of the waves, the situation will remain rather obscure. On the other hand, facts relative to the nature of the timing mechanism may be available from other sources.

The spermatozoa of a sea urchin (*Psammechinus*) exhibit two interesting features: (1) the tail is motionless when isolated from the "middle piece," and (2) a wave cannot pass beyond an element which is mechanically prevented from moving (Gray, 1955, Fig. 12e). If these two facts are of general application, there seems no need to postulate internal structures specifically concerned with the maintenance of phase differences between adjacent segments. As shown by Machin (1958) the form and rate of movement of the waves can be visualized partly in terms of passive conduction along an elastic filament and partly in terms of energy being fed into the system when elements undergo passive changes in length or tension. On this basis, uniplanar bending of an element near the proximal end of the tail would induce a wave traveling in the same plane, whereas if the proxi-

mal elements bend simultaneously in two planes, distal elements might also be excited to bend in corresponding planes. In other words, the bending waves would be comparable with muscular movements of "myogenic" origin. The rhythmical activity of sperm "models" when activated by ATP makes it difficult to think that anything comparable with conducted nervous impulses is fundamentally concerned, but here again the situation would be clarified by more precise information of the extent to which the form and frequency of the waves displayed by such models differ from those seen in a living flagellum (see Bishop, this symposium).

If the form and frequency of the waves are dependent on external forces, a relatively simple explanation is forthcoming for the fact that when two or more flagella are in close proximity with each other, their waves become synchronized; this phenomenon is, of course, not restricted to flagella, but probably occurs in all undulating organisms under comparable conditions. If, during its transverse movement an element of one individual came into contact with a more slowly moving element of another individual, its speed would be reduced while that of the latter increased. Sometimes an author uses an unfortunate simile. In discussing the relatively low metabolic level of a dense suspension of sea urchin spermatozoa (1928), I used the expression "They do not make any effort to move." This has been criticized by Rothschild (1961) as implying "some sort of free-will on the part of the spermatozoon." My remark was intended to convey the belief that isometric activity of the contractile elements of the tail did not occur. The evidence presented above supports the view that unless the tail is free to alter its shape, there is no conversion of chemical into mechanical energy along its length, and the metabolic activity may be expected to be correspondingly reduced.

Not the least interesting aspect of flagellar movement is one touched on by Rothschild (1961), namely, the very small dimensions of the filaments which exhibit it. Can it be that the whole of the essential structures may, one day, be located in a relatively small number of macromolecules each able to exhibit active changes in shape and able to liberate mechanical energy when subjected to external mechanical deformation? In this respect the work of Astbury's school may be of fundamental significance (Pautard, this symposium).

FORCES EXERTED BY FLAGELLA

The efficiency of a propulsive system can be assessed by the amount of useful work performed per unit expenditure of fuel. There seems no immediate prospect of reaching such an estimate for flagella by direct observation although the work of Yoneda (1960) on the forces exerted by a single cilium might perhaps form a basis for reasonable approximation. Calculations of forces or of work done, which are based on the assumptions at present underlying the general theory of propulsive speeds, must be accepted with caution; they are not only suspect for the reasons already alluded to, but also because they depend on the absolute value of the tangential drag coefficient about which there is considerable uncertainty.

An alternative approach to an estimate of the forces involved in flagellar activity might perhaps be derived from a study of the direction and velocity of displacement of very fine particles in the vicinity of the moving filament, in other words, from an extension of the earlier work of van Trigt (1919) and Lapage (1906). How far, if at all, such observations might be expected to lead to quantitative conclusions must be judged by hydrodynamical experts.

Experimental measurements of useful propulsive effort would be of limited value unless accompanied by information concerning the amount of chemical energy necessary to maintain a given amount of mechanical effort. The accuracy of any such estimate might well depend on whether the amount of metabolic activity necessary to maintain mechanical effort is large or small compared with that necessary to maintain other active processes within the cell which are not directly concerned with movement. These are problems for biochemists.

These rather discussive remarks might not inappropriately end where they began, by emphasizing flagellar movement as a most happy and fruitful field for cooperative effort.

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Sperm Movement
Problems and Observations[*]

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The speed at which a mammalian spermatozoon moves makes it difficult to see any details down the microscope with the naked eye. The alternatives are high-speed cinematography or slowing down the sperm movement. A film was made when the movements of the spermatozoa were slowed by increasing the viscosity of the suspending medium from its normal value of about 1 centipoise to 3000 centipoises. The film showed that, under these experimental conditions, bull spermatozoa move by passing two-dimensional waves of bending from the front to the back ends of their tails. The amplitude is not constant, nor is the wave form sinusoidal. In the film one spermatozoon, or spermatozoa, were seen moving faster than the others and appeared to have two tails. Possibly this was a monster. More probably it was two spermatozoa, whose tails were beating in phase, on top of each other. If the characteristics of the tail waves, which are called motility parameters, are unaffected by such proximity (which seems to be so in this case), one might expect, from hydrodynamic considerations, that two spermatozoa in such a position would travel faster than one alone (Taylor, 1951). Whether there is any reason for sperm tails to get into phase when close to each other is a question which will be discussed later.

MORPHOLOGY OF SPERM MOVEMENT

Surprising as it may seem, there does not seem to be universal agreement in 1960 as to whether the waves which pass along the tail of a bull spermatozoon are planar, that is to say two-dimensional,

or helical, that is to say three-dimensional. There is no doubt that the bull spermatozoa swimming in the unusually viscous medium passed two-dimensional waves along their tails, so that we can say that bull spermatozoa do sometimes swim in this way. But, for photographic purposes, a cover slip was put on the drop of sperm suspension. This means that the spermatozoa were in an abnormal environment, in the sense that helical waves might have been interfered with by there being too little distance between the microscope slide and the cover slip. According to van Duijn (personal communication), bull spermatozoa *always* propagate helical waves along their tails when the distance between the microscope slide and the cover slip is more than 40 microns. Rikmenspoel had, I think, the same view in 1957. I am certain this statement is not true when bull spermatozoa are examined in media with a viscosity of 2000–3000 centipoises. I have no apparatus with which to decide whether the waves are planar or helical when they are in their normal medium, at 37°C. I may have contributed to the differences of opinion about helical and planar waves by misinterpreting photographs of the apparently spiral tracks of sea urchin sperm heads under dark-ground illumination (Rothschild and Swann, 1949). Having drawn attention to this misinterpretation and having said that the waves which travel along sea urchin sperm tails are two-dimensional, Gray (1955) went on to show that, while propagating such planar waves, sea urchin spermatozoa rotate at a frequency of about two revolutions per second, whereas the tail wave frequency is twenty times as great. There is, of course, a difference between a planar wave, which periodically alters its plane relative to fixed axes, and a helical wave. The equations characterizing the two types of motion are, in cylindrical coordinates r, θ, x

$$r = b \sin k(x + V_w t) \tag{1}$$

$$\theta = \omega t$$

and

$$r = K \tag{2}$$

$$\theta = k(x + V_w t)$$

where b is amplitude, $k = 2\pi/\lambda$, λ is wavelength, $V_w = \nu\lambda$, the velocity of waves relative to the sperm head, ν is frequency, $\omega = 2\pi\nu$,

the frequency of rotation, and K is a constant. It would be interesting to compare the spatio-temporal geometry of the contraction-relaxation sequences in the fibrils of the sperm tail necessary to effect displacements of both types, that is, make a working model. The difference between the arrangement of fibrils in the mammalian and invertebrate sperm tail may be connected with the alleged inability of some of the latter to propagate helical waves. In mammalian sperm tails there are two concentric rings of nine fibrils. In invertebrate sperm tails there is only one ring, each member of which appears to be double because each fibril is divided into two halves by a septum.

Bishop's observations (1958) on squid spermatozoa are analogous to those of Gray, in that in both cases rotation about the long axis is said to occur. But if I have understood him correctly, Bishop said that squid spermatozoa can rotate about their long axis without passing bending waves along their tails—he refers to "straight, otherwise motionless" spermatozoa (1958, p. 1638). How do they manage to rotate without passing spiral waves along their tails or planar waves which periodically vary the angle between the plane of vibration and fixed reference axes? I can understand why the spermatozoa might not move forward and in this sense be motionless, but not how they can rotate with a straight tail. Perhaps Dr. Bishop will explain this point. In the same paper Bishop (1958) reports a most interesting observation, that tired squid spermatozoa can be rejuvenated by the addition of adenosine triphosphate (ATP) to the sea water round them. At the time, this was and, probably, still is the only proven case of ATP exerting its classical intracellular function when applied outside the cell membrane. ATP has, for example, no effect in restoring the cyanide-poisoned sodium pump in the squid giant axon when added to the surrounding sea water. But when injected into the axon, the poisoned pump starts up again (Caldwell et al., 1960). I suppose spermatozoa have a normal cell membrane? Apart from the usual black line or two seen at the sperm surface in electron micrographs, is there any evidence that they have? The fact that bull spermatozoa do not seem to shrink or swell in anisotonic media (Pursley and Herman, 1950; Rothschild, 1959) may indicate that they have an atypical cell membrane. Could someone measure the electric capacitance of the membrane to see if it is 1 $\mu f/cm^2$?

Unfortunately, spermatozoa are rather an awkward shape for such measurements.

To return to the morphology of sperm movement, things are less clear in the bull than the sea urchin spermatozoon. When swimming, at any rate under optimal conditions, the head often rotates or oscillates; this is easy to see only because the bull sperm head is shaped like a thin elliptical disk, which makes it look quite different down the microscope according to its orientation relative to the optical axis of the microscope. One might easily miss the rotation or oscillation of the sperm head in cases where the head is pear-shaped. Partly because of this rotation or oscillation, the suggestion has been made that the distal part of the tail propagates a helical wave, whereas the anterior part does not. This suggestion was, I think, first made by Gray (1958), who also raised the question whether the distal part of the bull sperm tail might not be twisted. The helical wave proposition has been faithfully repeated by other workers in the field, though, as mentioned earlier, there is not much objective evidence for the existence of helical waves in any part of the bull sperm tail and some evidence to the contrary.

One observation many gametologists must have made on bull spermatozoa is rather difficult to interpret: if one makes dead bull spermatozoa flow along a tube, one can often see their heads flashing, oscillating, or rotating in the way live ones do. Does this mean that the whole spermatozoon is rotating or oscillating, or can the head do so by itself? There is a curious structure in the neck of the bull spermatozoon, Fig. 1, and, for that matter, in that of man, which sometimes looks like a pile of circular disks, though its geometry has not yet been completely elucidated. Can the bull sperm head oscillate or rotate around these disks independently of the tail? Of course, a torque must be applied to effect such a rotation. But where does the torque come from when a dead bull spermatozoon is flowing along a glass tube? Perhaps someone should make a large model of a bull spermatozoon, drop it down a tube of treacle (molasses), and see what happens. One must use treacle, because a real spermatozoon thinks it is swimming in treacle, not water, when in its normal medium. This experiment would, of course, bring us into the realm of hydrodynamics or fluid dynamics, about which I would like to say a few words.
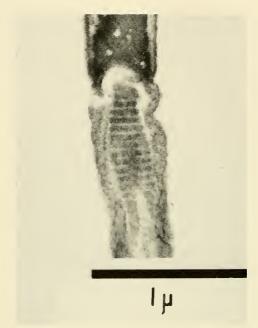
FIG. 1. Sagittal section of neck of bull spermatozoon. Fixation, 40% OsO_4 in carbon tetrachloride, one hour. Araldite embedding ($\times 51,000$).

HYDRODYNAMICS OF SPERM MOVEMENT

Some of us have amused ourselves by calculating the amount of energy a spermatozoon expends in swimming and relating the value so obtained to the amount of free energy available from the breakdown of exogenous substrate or the hydrolysis of ATP. The two are not the same, but the party line is to concentrate on ATP, the excess free energy transferred during fructolysis being assumed to be useless, wasted, or used for some hypothetical maintenance. Weber, in his first Dunham Lecture at Harvard (1958, p. 4), said, "Since all the energy provided during the course of metabolism is finally transferred to ATP, . . . ". This statement is difficult to accept in the case

of bull spermatozoa, as the free energy transferred during anaerobic fructolysis is 47.3 kcal/mole, which even the most confirmed optimist would agree is more than the terminal phosphate bond energy of 2 moles of ATP. As a matter of fact, it would be very silly of bull spermatozoa to use all the free energy of fructolysis to synthesize ATP, because it they did, the reaction

$$\text{Fructose} + 2P_i + 2\text{ADP} \rightleftharpoons 2 \text{ lactate}^- + 2\text{ATP} + 2\text{H}^+$$

would be in equilibrium, and the net rate of fructolysis would be zero. Paradoxically, therefore, it is useful and necessary to "waste" some of the free energy of fructolysis. I referred to calculations of sperm energy expenditure as an amusement, and I must confess to having experienced a feeling of mild amusement when finding that, in spite of being so different in regard to their motility parameters and metabolism, a bull and a sea urchin spermatozoon expend the same amount of energy, 2×10^{-7} erg per second (Rothschild, 1961). Such calculations are of questionable value because we do not know the effect on sperm A's energy expenditure of a nearby sperm B, whether it is motionless or not. The total energy dissipated by two spermatozoa beating in phase may be less than if they are out of phase, though even this is not certain because Taylor's analysis of this problem (1951) applied to infinitely wide waving parallel sheets, which are not the same as parallel cylinders, let alone nonparallel ones. Even if the problem is simplified in the following way, no answer is available from our hydrodynamic advisers, though perhaps Dr. Carlson will be able to dispel some of the gloom which surrounds the following apparently simple problem. Suppose an inertialess rod, or better still an inertialess rigid metal strip, oscillates in a viscous medium with fixed frequency and amplitude. One can measure how much energy is needed to make the strip behave in this way. Now suppose one puts into the medium another, stationary strip, free to oscillate, of the same size, fairly near the oscillating one, for example one strip length away. How much more energy has one got to pump in to keep the first strip oscillating in the same way as before the other, stationary strip was put in? This is, of course, a grossly oversimplified system, both as regards strip density, separation, and behavior, in comparison with the one in which we are really interested. Yet the hydrodynamagician cannot answer the question.

He will, of course, say that the presence of the stationary metal strip will increase the amount of energy needed to keep the oscillating strip going; but unless the strips are ridiculously far apart, that is all he will say. If asked whether he has any other helpful observations, he may mention that there is no reason why a system of oscillating strips should get into phase and that *there is no reason why such a system should get into a condition of minimum energy expenditure.*

It is for these reasons that estimates of sperm energy expenditure and attempts to correlate them with chemical events, such as the breakdown and resynthesis of ATP in the sperm tail, are at present of little value, though, as mentioned before, it is difficult to resist doing the sums. One thing is fairly certain: under anaerobic conditions, there is not much energy to spare. If oxidative phosphorylation is allowed to occur, there is plenty of energy, unless one artificially increases the density of the suspension and, therefore, the viscosity of the system, or the viscosity of the suspending medium.

ATP, ETC.

It has been known for a little under twenty years that ATP is involved in sperm motility, but measurements of ATP have so far been confined to just ejaculated spermatozoa, very tired spermatozoa, or motionless ones. I thought it might be of interest to see how the ATP, adenosine diphosphate (ADP), and adenosine monophosphate (AMP) content of bull spermatozoa varied under anaerobic conditions and, at the same time, to measure their activity by the impedance change frequency method (Rothschild, 1948) and heat production (Rothschild, 1960). Neither of these methods actually measures activity but some property associated with activity. When doing these experiments, I was, therefore, once again reminded of the importance of developing an objective way of measuring sperm activity at physiological densities, something which, so far, has defeated us, unless the Rikmenspoel (1957) method meets the situation, which is doubtful because it involves a rather severe dilution of the semen. Although it is true that when bull spermatozoa have stopped swimming their ATP content is reduced, no detailed information about bull sperm nucleotide metabolism has, so far, been published. In experiments I am doing in collaboration with Dr. A. A. Newton, with

whom the results will be published, the changes with time in the concentrations of ATP, ADP, and AMP + IMP in anaerobic bull spermatozoa have been measured. The anaerobic heat production and impedance change frequency were measured at the same time. Large and characteristic changes occur in the rate of heat production of anaerobic bull spermatozoa without any corresponding changes in nucleotide concentration, and the same applies if one compares them with impedance change frequency. A further point of possible interest is the high concentration of ADP in comparison with that of ATP. This is characteristic not only of anaerobic bull spermatozoa but also of just ejaculated spermotozoa. Can someone say, for certain, whether bull spermatozoa are anaerobic or aerobic before ejaculation?

The heat production of anaerobic bull spermotozoa is caused by the combustion of fructose to lactic acid and the partial neutralization of the lactic acid by the seminal plasma. Useful work associated with this reaction is ultimately degraded to heat by the tails of the spermatozoa. As mentioned earlier, the amount of energy upon which the spermatozoa can, ideally, draw for movement is the free energy change, not the enthalpy change, associated with the reaction. The two values are very different, the former being 47.3 kcal/mole, and the latter 27.7 kcal/mole.

RHEOTAXIS

A different aspect of sperm movement which has recently intrigued me and which I believe may throw some light on the mechanism of sperm movement concerns the idea, often asserted in the nineteenth century, that spermatozoa swim upstream. Upstream is, of course, a vague phrase, about which I shall be more precise a little later. Walton believed at one time (1952) that these nineteenth century assertions were based on errors of observation and, perhaps, on wishful thinking, it being thought that because eggs came "down" the oviduct, there must be a flow of fluid down it and that spermatozoa would not reach the eggs in time unless they had swum upstream. In tackling this problem, the first task is to devise a controllable method of flowing a sperm suspension along a container and to be able to characterize the velocity gradient. The best system is one similar to a Couette viscometer, with the velocity gradient in the

vertical direction. This can be achieved by putting a sperm suspension on a glass plate, with a rotating glass disk in contact with the top surface of the suspension. The advantage of this system is that the velocity gradient is constant $r\omega h^{-1}$, where r is the radius, ω is the angular velocity, and h is the distance between the disks. Unfortunately, it is difficult to achieve the required velocity gradients, without rotating the upper glass disk at inconveniently high speeds. This, coupled with quite serious technical difficulties—the bottom and top glass plates must not be more than about 180 microns apart—led me to abandon this system and examine the behavior of bull and human spermatozoa in vertical parabolic velocity gradients, of the form $-2Uz/a^2$, where U is the velocity at the center of the container, z is the vertical distance from the center at which observations are made, and a is half the depth of the container, the width and length of the container being to all intents and purposes infinite. I thought it convenient to start by examining the behavior of dead spermatozoa. These behave in an interpretable way. In a vertical parabolic velocity gradient, dead bull spermatozoa point downstream in the top half of the gradient and upstream in the bottom half. The explanation is as follows. Because their average specific gravity is greater than that of the suspending medium, bull spermatozoa slowly sink. But the head is more compact than the tail and has a greater specific gravity (Kihlström, 1958), so that the spermatozoa sink head first. This means that in the top half of the velocity gradient, the head gets pushed forward, whereas in the bottom half, the tail gets pushed forward. One may, therefore, ask the question: "As, presumably, the specific gravity of the head of a live bull spermatozoon is also greater than that of the tail and as the average specific gravity of a bull spermatozoon is greater than that of seminal plasma, do bull spermatozoa always swim downward and, if not, how do they stop themselves from doing so?"

It was of interest to see how a dead spermatozoon turned around when the direction of the velocity gradient was reversed. Figure 2 shows how this happens in a just reversed velocity gradient, the spermatozoon having been orientated in the "wrong" direction just beforehand. The rotation, which is partly in the vertical direction, is around the head.

So much for dead spermatozoa. If we now turn to live ones, it is

Direction of flow

100 microns
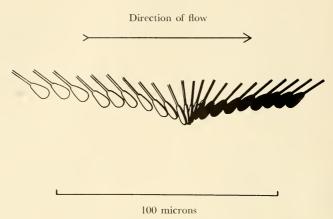
Fig. 2.   Motion of the head of a dead bull spermatozoon in a parabolic velocity gradient. The direction of flow was reversed 2.4 sec before the start of the record. Individual sperm heads are one-eighth sec apart. Rate of flow, 43 μsec^{-1}, 15μ from bottom of cell; velocity gradient, 2.6 sec^{-1}.

hardly necessary to say that the position is more complicated and different. To begin with, there is no doubt that both bull and human spermatozoa swim upstream in a parabolic velocity gradient, if the gradient is within the range 2–16 sec^{-1}. It is not at all easy to understand how a spermatozoon can swim upstream in both halves of a parabolic velocity gradient. If you throw me into a river at night, there is no way by which I can tell whether I am swimming upstream or downstream once my velocity is constant. One device for measuring the stress on the front of my head would not help, though, assuming I am the size of a spermatozoon, two tangential stress gages and a speedometer would do the trick. There is no known object, including pathologically shaped idealizations, which will point in the same direction in both halves of a parabolic velocity gradient. Suggestions as to how a spermatozoon does it would, therefore, be appreciated. A so-called simple explanation, recently put forward by Bishop and Walton (1960), that the tail offers less "resistance" to the flow than the head and is therefore carried downstream quicker is fallacious.

"9 + 2"

Those interested in flagella and cilia have spent quite a time in trying to think of some explanation of the ubiquitous 9 + 2, or 9 + 9 +2, pattern of fibrils. Unless one assumes that the preparative techniques for electron microscopy burn up and distort the original structures to an extent which is scarcely credible, one must dismiss the "commutator hypothesis" first put forward by Manton and Clarke in 1952, and repeated in 1955 by Astbury *et al.* Nor does the recent effort at explanation, if that is the right word, by Serra (1960), have much to commend it. He says, for example, that one explanation of 9 + 2 requires that the fibrils should follow a helical path along the cilium or flagellum. In fact this has nothing to do with 9 + 2. The advantage of a helical arrangement of fibrils is that, if it exists, bending waves can be propagated along the flagellum without the need for individual fibrils to contract and relax along their lengths. As only one spiral is needed along the whole flagellum, it is not easy to disprove that such spiralization occurs. Figure 3 is an electron micrograph of a longitudinal section of a bull spermatozoon in which the fibrils obviously follow a straight and not a spiral path; but it is only a few microns in length, whereas the whole tail is fourteen times as long. If lower magnifications were used so that longer lengths of the tail could be examined, the fibrils would be difficult to see. Serra goes on to say (1960, p. 396) that the movement of flagella and cilia "so far as is known always takes place by bending in a plane perpendicular to that of the 2 central fibres." Is such information available about any spermatozoon? I think not. In the bandicoot spermatozoon, I believe that the bending waves are in the *same* plane as the two central fibrils (Fig. 4), though Cleland, with whom I studied the ultrastructure of the bandicoot sperm tail (Cleland and Rothschild, 1959) does not agree with my interpretation of films of swimming bandicoot spermatozoa. If I am right, the role of the peripheral fibrils in causing bending waves becomes rather obscure, because the fibrils concerned, numbers 1 and 6, are atypical in not being connected to the axial ring fibrils, whereas all the other peripheral fibrils are.

I only mention these two points in Serra's paper because there may be a danger of making generalizations about sperm movement
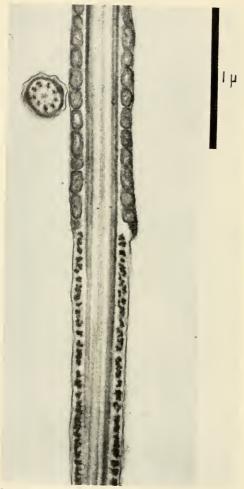
FIG. 3. Longitudinal section of bull spermatozoon through middle piece and part of tail. Fixation, 1% OsO_4 in veronal acetate buffer, one hour. Uranyl acetate staining, araldite embedding ($\times 47,000$).

on evidence which is neither extensive nor conclusive. Another ex-
ample concerns the actin-myosin interdigitation hypothesis of skeletal
muscle contraction. In at least two papers on sperm movement, the
suggestion has been made that, somehow or other, the fibrils in a
sperm tail interdigitate in a way analogous to that which possibly
occurs in skeletal muscle, thereby contracting and producing bend-
ing waves. Leaving aside that the fibrils in a sperm tail are not in
hexagonal array, are not attached to anything at their distal ends,
and not made of actin or myosin, why should a sperm tail work in
the same way as voluntary muscle, which has a different job to do,
not unnaturally done in a different way? At the moment, it is more
a question of ingenuity than reasoning to think of explanations or
mechanisms which might be consistent with the $9 + 2$ pattern.
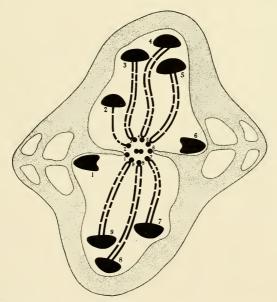
Fig. 4.   Diagram of a transverse section of the bandicoot (*Perameles
nasuta*) sperm tail. The dorsal and ventral halves of the tail are, respec-
tively, at the top and bottom of the diagram. The axial ring fibrils are 275
A in diameter. (After Cleland and Rothschild, 1959.)
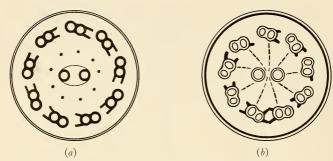
FIG. 5. (a) Diagram of a transverse section of a flagellum of *Trichonympha*. (After Gibbons and Grimstone, 1960.) (b) Diagram of a transverse section of the sperm tail of *Psammechinus miliaris*. (After Afzelius, 1959.)

I wondered, for example, whether the "arms" which are now being seen on the "clockwise" side of the fibrils in some sperm tails and cilia (Fig. 5) might be devices by which fibrils embrace one another, for some unknown reason, which implies fibrillar movement in a plane at right angles to that normally postulated. Alternatively, the number 9 may not have any particular point in regard to flagellar or ciliary movement and may have been developed for quite different reasons to do, for example, with cell division. The marked difference in the size and mode of anterior attachment of some of the bull sperm tail peripheral fibrils, recently demonstrated by Blom and Birch-Andersen (1960), which I have recently confirmed, may lend some slight support to this possibility. I have a working model of a spermatozoon at Cambridge, unfortunately too big to bring with me, which propagates bending waves from the front to the back end of its tail in an enjoyably spermlike way, with only two contractile fibrils in its tail. Of course—at least I think of course—the waves have to be planar if there are only two fibrils; if the waves were helical, or if their plane varied, one would need more fibrils.

One way to gain further insight into the mode of action of the fibrils and, therefore, of their raison d'être, might be by trying to observe them during activity. Given that in the nineteenth and early twentieth century zoologists were apparently able to see sperm tail fibrils with the light microscope, should we not have another shot

at doing the same ourselves, using dark-ground illumination? If, for example, someone were prepared to subsidize it, I would like to commission a tremendously powerful electronic flash lamp for use with the light microscope. Even with a small mercury arc lamp, I recently saw some "bacterial flagella," about 0.1 micron in diameter, on the surfaces of tick spermatozoa which, as some of you know, were, until recently, described as moving exclusively backwards, without any means of propulsion. Actually, their "bacterial flagella" do not propel them. As an alternative to the electronic flash program, which would also require electrical stimulation of fibrils or the use of sperm models, is it ridiculous to consider the possibility of freezing sperm bending waves and examining tails and their fibrils both with the light and the electron microscope, to see if there is any geometrical difference between a contracted and a relaxed segment?

These ideas may seem fanciful and are no doubt wide of the mark. But would there not have been similar scepticism ten years ago at the idea of "seeing" ATPase on peripheral fibrils with the electron microscope, as Nelson (1958) has done?

CONCLUSION

Why are we interested in sperm movement? Impressive answers to this question could and doubtless will be given by our distinguished speakers. A suspension of spermatozoa is one of the nicest assemblages of cells with which one could work. They respire—at least I think they do—though John MacLeod may have different views about human spermatozoa; they glycolyze, though they usually fructolyze; they have all the gadgetry which in other types of cells is usually examined in slices of organs or with the aid of that instrument so dear to the biochemist, the homogenizer; they contain interesting compounds and enzymes to help them in doing their duty toward the egg; parts of them are like, or are, muscle fibers so that sperm tails may help us to learn how muscles contract; parts of them are antigenic, which may help us to find out whether their tails contain actomyosin, something-myosin or a new "muscle" protein; they are easy but sometimes frustrating game for the electron microscopist; they move in an obvious way, unlike most other cells, and therefore tell us, visually, if they are alive (always assuming that if they don't move, they are dead); they are concerned with some of the most im-

portant problems which affect or afflict the world—overpopulation and sterility; finally, as their front end consists almost entirely of DNA, they have the right amount of glamor to enable us to get the support to continue our work.

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Biophysical Approaches to the Measurement of Sperm Motility

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The parameters of sperm motility and their methods of measurement have already received considerable attention by a number of investigators (see also Bishop, 1962). Essentially, two fundamental kinds of problems are involved in the concept of sperm motility, those associated with the nature of the mechanism of movement of individual cells, and those concerned with the measurement of relevant statistical quantities which characterize the "motility status" of a semen sample.

The experimental approach to each of these kinds of problems will, of course, vary. For detailed observation of individual movements, cinemicrographs of semen samples seem to be the best means available. Measurement of statistical quantities, such as the mean velocity and concentration, can be attempted only after establishing that sperm cells obey appropriate statistical laws. This, too, is facilitated by means of cinemicrography. This paper will be devoted to an account of recent experiments performed to elucidate some aspects connected with both sets of problems involved in sperm motility.

SOURCE AND HANDLING OF MATERIAL

Semen was obtained from bulls at the experimental farm of the Research Institute for Animal Husbandry at De Bilt, Holland, and from the Southeastern Pennsylvania Artificial Breeder's Cooperative at Lancaster, Pennsylvania.

After ejaculation, the semen was diluted ×10 in optically clear,

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15% egg yolk citrate buffer (Rikmenspoel, 1957a). During transporta-
tion the semen was cooled to 4°C. Then a second dilution of 6 to 20×
was performed to make the concentration of the final suspension
suitable for measurements.

If not mentioned otherwise, the standard conditions during meas-
urements were: pH = 6.7 ± 0.1, T = 37.1°C, freezing point de-
pression Δ = −0.54 ± 0.02°C.

OBSERVATIONS ON MOVEMENTS OF INDIVIDUAL SPERM CELLS

Apparatus

Most of the data reported here were obtained from moving films
taken at a speed of 50 frames/sec. Because of the special characteristics
of this apparatus, certain features should be specified (Rikmenspoel
and Van Herpen, 1956). Dark-field illumination was obtained by
means of a condensor with central stop. The light source was a
specially developed electronic flash bulb giving an exposure time
for each frame of about 150 μsec (Robinson, 1954). This short ex-
posure per frame was sufficient to avoid blurring of the images of
the sperm due to movement. A Reichert achromatic objective, ×10,
NA = 0.25, and a Reichert projection eyepiece were used, the latter
adjusted so as to give a magnification from sample to emulsion of
×20. A measuring chamber for the sperm sample 42 ± 2 microns
deep was used; this consisted of two slides each 1 mm thick, separated
by a plastic foil perforated by a hole 15 mm in diameter. About 40
microns should be considered the minimum depth of such a cham-
ber, if interference with the "vertical" tail movement is to be
avoided. The length of each film was about 30 sec. This is a suf-
ficient period to measure statistical data such as *concentration* and
velocity distribution of one sample (see below).

Description of Movements of Sperm Cells

Films obtained were projected frame by frame, which permitted
the consecutive positions of individual sperm to be drawn on paper.
By neglecting motionless cells and those which are clearly pathologic
(due to broken tails or strongly curved middle pieces), the cells
were found to fall into two distinct classes:

1. Cells which rotate along their longitudinal axes, while moving

in fairly straight paths. The heads of these sperm show periodic light flashes in dark-field illumination but not in phase contrast.

2. Cells which swim in small, almost closed, circular paths. These sperm do not rotate and show no flashes off their heads.

These two types of motile sperm will be considered in some detail.

Rotating Cells

The successive images of three cells are shown in Fig. 1. The flashes off the heads are indicated by the fat, black heads. In these positions the cell lies with the flat side of its head (in bull sperm, a flat elliptical disk), perpendicular to the slide surfaces. The light beams of the dark-field illumination are then reflected into the aperture of the objective. In all other positions, light is scattered off the edges only of the head, so that a ring is seen. This phenomenon is clearly due to a rotation of the whole cell along its longitudinal axis.

The wave which progresses along the tail of these cells is, remarkably, synchronous with the rotation. This is seen in Fig. 1, where, after a complete rotation, the whole cell returns to its original position. The alignment of the tail with the head whenever it flashes,
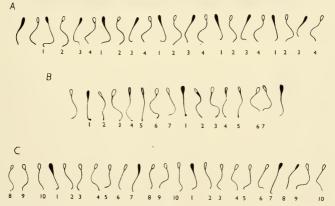
Fig. 1. Successive positions of three rotating cells (A, B, and C respectively) at intervals of 1/50 sec. For each of the cells the positions are numbered; after each period of rotation the same sequence of positions recurs.
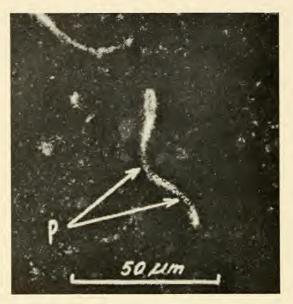
Fig. 2.   Enlarged reproduction of the image of a rotating cell; at the points *P* the tail has moved out of the plane of focus.

as shown in Fig. 1, has also been observed by Gray (1955). The tail wave of the rotating cells is three-dimensional. Figure 2 illustrates a rotating cell where the image of the tail is blurred in two places, owing to the fact that it has moved out of the plane of focus.

An attempt was made to measure the "vertical" amplitude from the amount of blur of the tail image. Figure 3 represents the measured width of the image on a film of the tail of a dead sperm cell as a function of the distance to the focal plane. Unfortunately no quantitative data can be obtained in this way, chiefly because a well-focused tail is blurred to about three or four times its diameter by spherical aberration from the 1-mm-thick cover slide and by the grain of the emulsion. Qualitatively, however, the fact that rotating cells have *a three-dimensional (helical) wave is firmly established.*

The forward movement of the sperm, measured as the distance
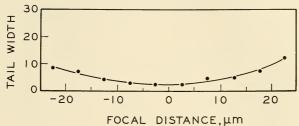
FIG. 3. Width of the image of the tail of a sperm cell as a function of the distance to the plane of focus. Width expressed in microns (μ and μm employed interchangeably in figures throughout paper).

covered during consecutive intervals of 0.5 sec, is constant within 4% (standard deviation) during the time the cells can be observed before leaving the field of view. The "orbits" of the cells are fairly straight; of 150 cells measured, 92 had a radius of curvature of the orbit $r >$ 1 mm, 36 had $1 < r < 0.5$ mm, and the rest $r < 0.5$ mm. The implication of this is that for statistical measurements, the *velocity* of a sperm and also the velocity distribution of a sample are useful concepts, and that random samples of the moving cells can be obtained.

The velocity turns out to be proportional to the frequency of rotation (Fig. 4). If we write:

$$v = p \cdot f_{rot} \qquad (1)$$

the constant p represents the pitch of the helical movement of the head. For the ejaculate represented in Fig. 4, mean and standard deviation of p are: $p = 12.6 \pm 2.4$ microns. From seven ejaculates* it was found that:

$$p = 11.9 \pm 0.7 \ \mu$$

Even though the tail wave cannot be expected to be sinusoidal or to have a constant amplitude as it progresses along the tail (Machin, 1958), an average value for the amplitude can be measured, so long as the tail, at the moment the data are taken, is "parallel" to the slide

* Most of these data were obtained by the method of dark field-track photography, as introduced by Rothschild (1953b). This procedure saves considerable time needed for analysis.

ROBERT RIKMENSPOEL
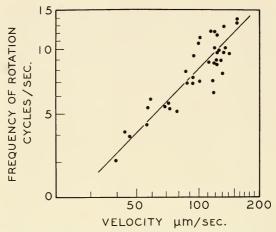
Fig. 4. Relation between velocity and frequency of rotation of spermatozoa.

surface. Table I includes data and the degree of accuracy obtained in this way. The velocity of a cell is related quadratically to the mean amplitude b of the tail wave (Fig. 5). If we write:

$$v = \varepsilon_{rot} \cdot b^2 \qquad (2)$$

it is found that

$$\varepsilon_{rot} = 1.2 \ \mu^{-1} \ \sec^{-1}$$

TABLE I. Amplitude of the tail wave, measured at several consecutive moments

Cell No.	Amplitude b of Tail Wave Measured at Several Moments (μm)					Mean Value of b and Standard Deviation (SD) (μm)	
1	11	7	10	10		9.5	1.7
2	9	10	7	11	8	9.0	1.6
3	8	5	7	8		7.0	1.4
4	9	11	10	12		10.5	1.3
5	7	8	5	6		6.5	1.3
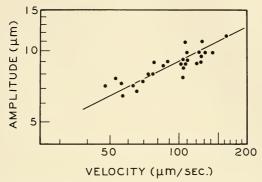
Fig. 5.   Relation between velocity v of rotating sperm and the amplitude b of the tail wave.

The constant ε_{rot} can be interpreted as expressing the efficiency of the tail wave in propelling the sperm. The frequency of the tail wave f could not be measured from these films since the frame sequence proved to be too slow for the purpose. Because the tail wave is synchronized with rotation of the cell, the ratio f/f_{rot} must be an integer; but, as can be seen from Fig. 1, it is not possible to decide whether this ratio is 1, 2, or 3.

Cells Swimming in Circles

Successive positions of a cell swimming in a circular orbit are shown in Fig. 6. These cells show no rotation, and the tail wave turns out to be flat, that is, two-dimensional. Within the accuracy of our observation, no "vertical" movement was measured.

The amplitude of the tail wave of these cells shows a clear asym-

Fig. 6.   Successive positions at intervals of $1/50$ sec of a sperm swimming in a circle; the orbit is shown at each position.
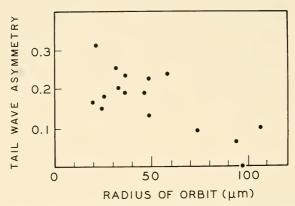
FIG. 7. Correlation between the asymmetry of the tail wave and the curvature of the orbit of sperm swimming in circles.

metry relative to the tangent of the circular orbit. This asymmetry, expressed as $(b_2 - b_1)/b$ (where b_1 is the amplitude toward the center of curvature of the orbit, b_2 the opposite amplitude, and b the average), shows some correlation to the curvature of the orbit, as shown in Fig. 7.

The mean amplitude b of the tail wave is again quadratically related to the velocity of the cell,

$$v = \varepsilon_{cir} \cdot b^2$$

and for ε_{cir} is found,

$$\varepsilon_{cir} = 0.8 \ \mu^{-1} \sec^{-1}$$

It is thus seen that the efficiency of the flat tail waves (represented by ε_{cir}) is less than that of the helical waves (see above).

The frequency of the tail wave can be measured for circularly orbiting cells, since no rotation is interfering with the picture. No correlation between this frequency and the velocity was observed.

Discussion Concerning Cell Movement

Of the two types of movement described, the rotating type is to be considered normal, and the nonrotating one as pathologic. The main

arguments in support of this view are: (1) the movements of the non-rotating, circular swimmers are asymmetrical; (2) rotating cells can be transformed into nonrotating ones by cold shock; (3) the velocity of circular swimmers is in general lower (20–100 μ/sec) than that of rotating cells (40–160 μ/sec); and (4) more or less intuitive arguments render illogical the concept that the healthy sperm limit itself to two dimensions and restrict itself in a process like fertilization (essentially a chance process of *meeting*) to an almost fixed position (the circular orbit).

It becomes now possible to define, in a more precise way, the "motility" of a semen sample. For normal, rotating cells *concentration, velocity distribution*, and *mean velocity* are useful concepts, which can be measured by methods involving a sampling procedure. The method for measuring concentration and velocity distribution as described in the next section, and that which depends on "probability after" effects (Rothschild, 1953a) are in fact based on the assumption that a small "subvolume" of sample shows random fluctuations with time. This will hold for the normal, rotating cells of a semen sample because the mean free path of such a sperm can be considered long and its velocity rather constant. Thus, by observing over a period of time a small part of a slide such as the field of view of a microscopic objective, a random test is obtained of concentration and velocities of the rotating cells. This is not true, however, for cells swimming in circles as they are practically bound to their closed orbits. The cells of this type which are present in a field of view at a certain moment will remain there, and no new ones will enter. Special care should thus be taken in interpretation of motility data obtained from sperm samples containing a large percentage of circularly swimming cells.

In the past, several attempts have been made toward a theoretical approach to flagellar movement. Taylor (1952) considered sinusoidal waves of small amplitude traveling along infinitely long flagella; it was found in this case that the flagella should have a velocity:

$$v \doteq \frac{f \cdot b^2}{\lambda} \tag{3}$$

where f is the frequency of the wave and λ the wavelength. When the wave is helical (three-dimensional) a torque g tending to rotate the

flagellum is exerted by the surrounding fluid:

$$g \doteqdot \eta \frac{f \cdot b^2}{\lambda}$$

where η is the viscosity of the fluid. From this it follows that under these conditions the flagellum rotates with a frequency proportional to the velocity. A flat tail wave would, of course, not induce any torque. The main observed properties of sperm movement are indeed described in this way: (1) the forward velocity is a second order effect, proportional to the square of the amplitude b, and (2) the helical waves give rise to rotation of the whole cell.

Later Gray and Hancock (1955) derived for a flat sinusoidal tail wave essentially the same formula as Taylor (equation 3), by using a simplified model of the fluid motion around the tail which enabled them to extend the theory for larger amplitudes.

The models of both Taylor and of Gray and Hancock appear too simplified to yield a quantitative description of sperm movement. Actual measured data for f, b, and λ of circularly swimming cells give, according to equation (3), velocities which are too high (Rikmenspoel et al., 1960), by a factor of 10 for the model of Taylor or a factor of 5 for that of Gray and Hancock.

DETERMINATION OF STATISTICAL DATA OF SEMEN SAMPLES

Apparatus

For measuring mean velocity of semen samples, a photoelectric device has been developed, the principle and design of which warrant some discussion. A semen sample is illuminated in dark field so that the sperm appear bright on a dark background. An objective focuses the sample on a diaphragm. An aperture in the diaphragm, about the size of the image of the head of a sperm cell, is viewed by a photomultiplier. The multiplier thus "looks" at a certain small area of the sample. Whenever a sperm cell passes over this area, the multiplier receives a light signal, which is recorded or electronically analyzed.

The specimen of semen is again 42 ± 2 microns deep, and a low power objective ($\times 10$, NA $= 0.25$) is used. The aperture in the diaphragm is 100 microns in diameter, representing 10 microns in the sample.

A recording of the passage of a rotating sperm cell is shown in Fig. 8. The flashes due to the rotation of the head are very clear; the "passage" of middle piece and tail can also be distinguished. A recording of the passage of a circularly swimming (nonrotating) cell is given in Fig. 9. The absence of flashes from the head and the regular movement of middle piece and tail conform to the picture of these cells obtained from the films.

When a sperm cell passes over the aperture, it holds, for the distance covered, during the time any part of it is visible, that:

$$v \cdot t = l \tag{4}$$

where v is the velocity of the cell and t the duration of the passage. The distance l is essentially equal to the length of the cell plus the diameter of the aperture. The cell is, however, virtually shortened because of the waving movement of the tail. Taking into account the
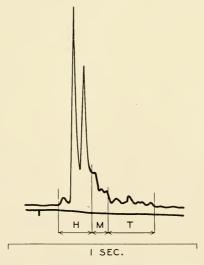
Fig. 8. Record of the passage over the aperture of a rotating sperm cell; the record can be divided into three parts, corresponding to the movements of the head (H), middle piece (M), and tail (T) when projected on the aperture.
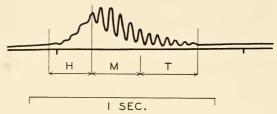
Fig. 9. Record of a nonrotating cell passing over the aperture; head and middle piece plus tail can be roughly distinguished.

relation (2), $v = 1.2 \cdot b^2$, and approximating the tail wave to be sinusoidal, the expected value for l can be calculated (Rikmenspoel, 1957b). The relation is shown in Fig. 10. A model experiment was performed to check this approximation; films were projected on a screen on which the aperture was represented, and l was measured of the sperm "passing" over it. These data are represented in Fig. 10. In view of the approximate nature of the calculated line, the slight discrepancy is not disturbing. An error of 10 to 12% (Rikmenspoel, 1957b) is to be expected as a result of several factors in measuring the velocity of a cell passage: (1) uncertainty in reading the beginning and end of passage, (2) blurring of the cell image by the limited
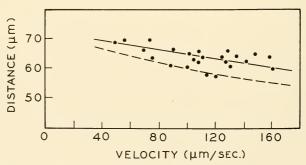
Fig. 10. Distance l covered by a rotating sperm during passage over the aperture, as a function of the velocity v of the cell. The dotted line represents the value of l from an approximate calculation.

depth of focus, (3) variation in cell length, and (4) passage off center of the aperture. With these factors in mind, the standard value of l adopted for interpreting the recordings was

$$l = 64 \, \mu$$

without taking into account the slight dependence of l on v.

For the rotating cells, which satisfy statistical sampling conditions,

$$n_v dv \doteq v \cdot f(v) dv$$

where n_v is the number of cells with velocities between v and $v + dv$ passing the aperture per second, and $f(v)$ is the velocity distribution of the sample. By recording passages over a long time interval, the velocity distribution and, thus, the mean velocity are obtained. Figure 11 shows the mean velocity obtained in this way versus the mean velocity determined from films taken on the same slide immediately after the photoelectric measurement was complete. An accuracy for measuring $\bar{v}$ of about 4% is obtained.
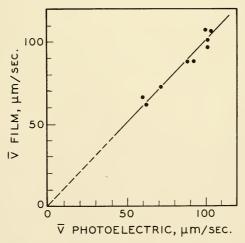
Fig. 11. Mean velocity of sperm samples measured photoelectrically and by means of films.

For the total number, n, of cells (of all velocities) that pass the aperture per second,

$$n = c \cdot \bar{v} \cdot Q \tag{5}$$

where c is the concentration of the sample and Q is a proportional constant. Since $\bar{v}$ is independently determined, equation (5) enables one to measure the concentration. The constant Q was calibrated by comparing the number of counts of a sample with the concentration measured from a film of the same slide:

$$Q = 15.5 \pm 1.2 \ \mu$$

The standard deviation in Q of 8% is caused by variation in sperm size, focusing errors, etc. Since, in general, the statistical spread of n is from 6 to 8%, the concentration can be measured in this way to about 10 to 12% accuracy.

Recently a signal analyzer was built, based upon the following principle: the time, Δt, between two light flashes of the head can be written,

$$\Delta t = p \cdot l / v$$

where p is the constant in equation (1). Whereas p has a scattering (standard deviation) of about 25% for different cells the average value of p has a much smaller spread of about 6% from one sample to another (see Rikmenspoel, 1957b, p. 67). Thus, useful data can be expected by equating:

$$v = p \cdot l / \Delta t$$

and taking for p the average value, $p = 11.9$ microns/sec.

This principle has been technically realized in the following way. By means of a discriminator, the spikes caused by the sperm head are transformed into short pulses. The first of these pulses sets into action a string of univibrators, the delay time of which is adjusted so that each represents a velocity class of 20 microns/sec interval. The delay times should be thought of as in series. A scale of 2 selects the second flash. Coincidence circuits determine with which univibrator this second flash is coincident; mechanical counters register the number of observed cells in each class. In this way a velocity distribution is immediately obtained. The total number of cells passing the aper-

ture is counted on a separate register. The aperture is now made somewhat larger (equivalent to 15 microns ϕ in the sample), to ensure that, in most cases, more than one spike of a rotating head will be seen. Provisions are made to (1) close the detector as soon as the velocity of a cell is classified, in order to prevent interference from an eventual third or fourth flash of the head, (2) reset the scale of 2 if only one flash is seen (e.g., by passage at the edge of the aperture), (3) close the detector if the background of the sample rises too high as when agglutinated cells or pieces of dirt drift by, and (4) measure the elapsed time the detector is open to give signals to the discriminator. In this respect the apparatus resembles somewhat that of Bosselaar and colleagues (1952, 1955).

Calibration of the accuracy of the apparatus was made by comparing the data with the absolute figures obtained from dark field-track photographs taken with an exposure time of 1 sec of the same slide directly after the electronic measurement was performed. The velocity of the sperm can be determined from the length of the track made during this time, after appropriate correction (see Rikmenspoel, 1957b, p. 43). The results of this calibration are given in Figs. 12 and
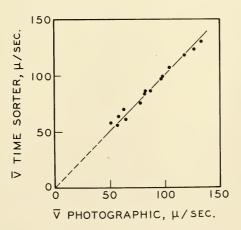
FIG. 12. Mean velocity of semen samples as measured with the electronic analyzer and from photographs.
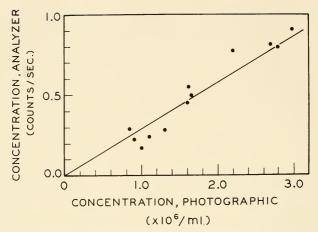
FIG. 13.   Concentration of semen samples measured with the analyzer and by photographs, respectively.

13 for $\bar{v}$ and c, respectively. The accuracy obtained for $\bar{v}$ is 6 to 7%, which agrees well with the scattering in p; c can be measured with an error of about 15%. This is larger than the error to be expected from statistics; in this respect the apparatus is inferior to the visual analysis of recorded signals as described above. For almost all other purposes, however, the accuracy of the electronic analyzer is adequate, and the time saved from the tedious work of visual analysis of recorded signals is considerable.

Some Measurements of Sperm Motility

With the apparatus described in the previous section the behavior of diluted bull semen was determined under various conditions. The main purpose of these experiments was to gain an understanding of the strictness of control of experimental circumstances required for motility measurements. The conditions of measurement were the same as mentioned above unless otherwise noted.

Natural Variation of $\bar{v}$.   The mean velocity of twenty-four ejaculates of four bulls was measured under standard conditions (Fig. 14). The average value of $\bar{v}$ is 97 ± 6 (standard deviation) microns/sec.

TABLE II. Mean velocity ($\bar{v}$) of normally moving sperm cells from four different bulls; no significant individual specificity

Bull	Number of Ejaculates	Mean Value, $\bar{\bar{v}}$, of $\bar{v}$ for the Different Ejaculates (μm/sec)	Standard Deviation of $\bar{v}$ (μm/sec)
D 24	6	96	6
A 10	5	97	5
R 2	4	102	6
W 1	6	94	6.5

This agrees well with an average figure of 94 microns/sec for 213 sperm cells reported by Gray (1955). No individual specificity in the bulls was observed, as can be seen from Table II.

Variation of $\bar{v}$ with Temperature. The temperature dependence of $\bar{v}$ was measured for thirteen ejaculates of four bulls in the range 33–43°C. A fresh slide was made for each measurement. Figure 15 represents
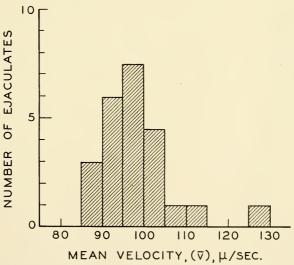
Fig. 14. Variation in mean velocity v of twenty-four ejaculates from four bulls.
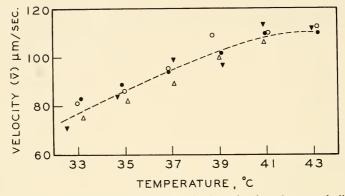
Fig. 15.   Temperature variation of $\bar{v}$ for four ejaculates from one bull.

the data obtained from four ejaculates of the same bull. In the range
33–41°C the temperature coefficient is approximately constant; an
optimum is apparently reached around 43°C. This was also observed
for sperm of other bulls. Statistical analysis (kindly performed by
Dr. B. de Groot), showed that the ejaculates from each bull could
be "pooled" in calculating a temperature coefficient for $\bar{v}$. These
coefficients are shown in Table III. Since the standard deviation of
$\partial\bar{v}/\partial t$ is about 0.5 micron/sec/°C, these values do not clearly indicate
individual specific coefficients for each animal's sperm.

TABLE III.   Average temperature coefficient for four different bulls in the
range 33–41°C

Bull	Number of Ejaculates	Temperature Coefficient $\mu/sec/°C$
D 23	4	4.0
W 1	3	3.8
R 2	3	3.4
A 10	3	2.2

Aging of Sperm Cells.   Samples taken at regular intervals from semen
stored at 4°C were used for measurements, at 37°C, of velocity $\bar{v}$ and
concentration c of normally moving cells. Both these properties were
found to decrease exponentially with time, as illustrated in Figs. 16
and 17. The half-life of c is much smaller, however, than that for $\bar{v}$.
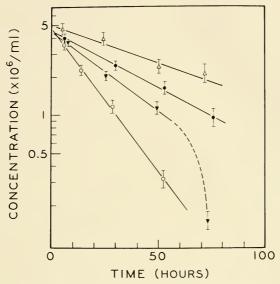
FIG. 16. Change with time of the number of normally motile cells from four ejaculates.
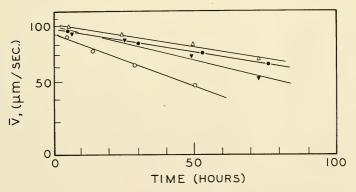
FIG. 17. Change with time of the mean velocity of the normally moving cells from four ejaculates.

TABLE IV.   Half-life for the decrease in number of normally moving cells during storage at 4°C

Bull	Number of Ejaculates Measured	$t_{1/2}$ (hours)
W 1	3	49 ± 9
D 23	4	51 ± 14
A 10	3	27 ± 3.5
R 2	2	14 ± 1

The half-life for the decrease in the number of moving cells shows a definite individual specificity for each bull (Table IV).

Dependency on pH. Although different ejaculates show a rather large variation in dependence of mean velocity of normal sperm on pH (van Duijn and Rikmenspoel, 1960), a sufficiently general picture can be obtained by averaging the pH over several ejaculates. This is shown in Fig. 18. The average increase over the pH range 5.7–7.6 was $\partial \bar{v}/\partial \mathrm{pH} = 10 \pm 1$ microns/sec/pH unit.
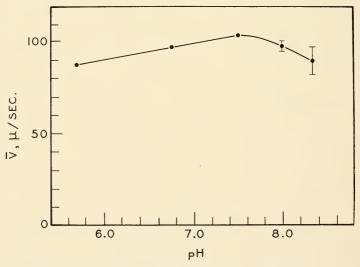
FIG. 18.   Dependence of $\bar{v}$ on pH, averaged for several ejaculates. (Courtesy of C. van Duijn, Jr.)

INTERACTION OF SPERM CELLS

Observations

It has long been known that suspended microscopic particles (e.g., erythrocytes, fat globules, bacteria) show a repulsive interaction (Berkson *et al.*, 1940; Van Kreveld, 1947). As a result, the standard deviation of the number of particles present at regular time intervals in a sample volume is found to be smaller than would be predicted by a Poisson distribution. On the other hand, theoretical evidence has been presented by Taylor (1952) for an attractive interaction between microorganisms with undulatory flagella, such as sperm cells. Taylor found that two flagella close together, having a synchronized tail wave, dissipate less energy into the fluid than two independently moving flagella. In undiluted semen, where sperm are close together, interaction can be observed as "wave formation." Rothschild (1949) based his "impedence change" method for evaluating motility of undiluted semen on this effect.

Measurements of sperm interaction in diluted semen have been performed in two ways, by using the cinematographic and the photoelectric methods described above. From films, the number of motile cells present on a slide in an area of 150 microns ϕ was counted at intervals of 2 sec. This interval is long enough to eliminate correlation between successive events. The standard deviation $\sigma(n)$ of the number of cells in the area, as illustrated in Fig. 19, is found to be

$$\sigma(n) = 0.93 \sqrt{n}$$

By means of the photoelectric device, time intervals between the beginning of passages of sperm were measured. If no interaction is present, the distribution $n(t)$ of interval lengths is exponential:

$$n(t) = \exp(-t/t_0)$$

where t_0 is the average interval. The standard deviation $\sigma(t)$ of the distribution then is $\sigma(t) = t_0$. Actually measured distributions are shown in Fig. 20; it is clear that the distribution is too low for the small intervals. For twelve ejaculates, it was found that $\sigma(t) = (0.94 \pm 0.2) \times t_0$, where t_0 ranged from 1.8 to 4.7 sec.

Discussion

The results of both types of measurements reported in the previous section indicate a small repulsive interaction. If the very crude
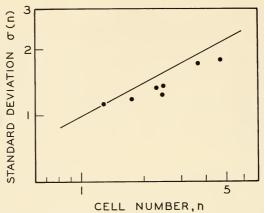
Fig. 19. Standard deviation $\sigma(n)$ in the number of cells present in an area 150 microns ϕ in various semen samples. The line represents the standard deviation for Poisson distribution.
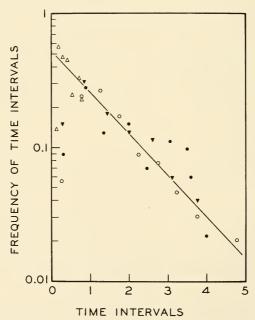
Fig. 20. Distribution of time intervals between passages of sperm cells over the aperture of the photoelectric apparatus. Four experiments. The data for the four ejaculates have been normalized so as to fit them to the same theoretical curve.

approximation is made to assign to a sperm cell a volume where another cell has no "access," this represents, in effect, a repulsive interaction. The spatial distribution in that case would be (de Vries, 1953)

$$\sigma(n) = [n(1 - 8k)]^{1/2} \qquad (6)$$

where k represents the fraction of the total volume occupied by the sperm cells. If a sperm is taken roughly as a cylinder of diameter a, and a length of 70 microns (the actual length of a sperm), then from equation (6) it would follow that $a \approx 8$ microns.

For the standard deviation of the distribution of time intervals, $\sigma(t)$ was calculated, again assuming the sperm cell to be a cylinder,

$$\sigma(t) = t_0 (1 - 0.026a/t_0)$$

From this, a value of $a \approx 3.5$ microns is obtained. In view of the approximate nature of these calculations, the difference of a factor of 2 in the values of a found with the two methods need not be surprising.

In conclusion it can be said that, in dilute suspensions of sperm, a weak repulsive interaction is present which prevents effects due to interactions as considered by Taylor (1952).

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Ionic Balance of Sperm Cells

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As you all know, conduction of nerve impulses and irritability in general are strongly associated with ion gradients, usually an outwardly directed potassium gradient, and an inwardly directed sodium gradient. All sorts of special pumps, binding mechanisms, and physiological gremlins have been postulated to account for this ubiquitous condition of all cells.

Unfortunately, ion gradients are so general in type and occurrence that it has been very difficult to decide just what physiological function causes the gradients and which functions depend upon them. It seems fairly clear that response to stimulation involves the energy of the gradient; presumably some metabolic system keeps the mechanism going.

It occurred to us that the nicely compartmentalized spermatozoon might provide some answers; there is here such a clear separation of the mobile, conductile tail, the metabolic midpiece, and the information-storage head. Furthermore, the compartments can be simply shaken apart and examined separately. We did just that and shall report some of the findings to you. [See Steinbach and Dunham (1961) for complete report.]

The general procedure was to collect sperm of three animals, *Arbacia*, *Mytilus*, and *Phascolosoma*, and treat them with appropriate solutions, usually sea water or mixtures of sea water and other solutions. We found we could easily fragment all three types of sperm into head-midpiece fractions and tail fractions by a few seconds treatment in a Waring Blendor, followed by orthodox differential centrifugation. Rather than make cell counts—after all we are both physiologists—we treated most samples with C^{14}-inulin to measure extracellular space. Centrifuged pellets of whole sperm, head midpieces, and tails were then analyzed by routine methods.

TABLE I.   Ionic concentration in *Phascolosoma* sperm

Fraction	% Total Volume	% Total Dry Weight	Conc., meq, wet weight		
			Na	K	Cl
Head midpiece	75	40	60	150	0
Tail	25	12	400	15	400
Sea water	—	—	420	10	510

The story can be made very short by reference to the data in Table I. Concentrations noted are approximate and are calculated on a wet weight basis. By our methods, head midpieces account for 75% of the total volume of the sperm and include most of the dry weight. Tails are very dilute in terms of dry weight. Head midpieces, separated from tails, retain the high ionic gradients of whole sperm. Tails act like so many highly permeable strands of jelly.

Our results could be explained by assuming that ion gradients are pumped up and maintained by the head-midpiece complex, which leaks ions of its characteristic pattern back through the tail. If this were true, head-midpiece fractions should have higher ion gradients than do whole sperm, since the leaking "parasitic" tails are now no longer present. With *Phascolosoma* sperm exactly this is found. Head midpieces have more K than do whole sperm; tails have virtually no excess. With *Arbacia* and *Mytilus* sperm, the results are less clear-cut. Inulin spaces increase markedly, and ion gradients fall in head-midpiece fractions. We suggest this is due to injury to some members of the population, the sperm of *Phascolosoma* being just a harder headed type.

With regard to mechanisms involved in establishing the gradients, we can offer little at the moment. There is no firm ion binding. Na and K of sperm are freely exchangeable as measured by isotope exchange with the environment.

Cellular K is retained tenaciously in the face of marked variation in total ionic strength of the environment with very slight osmotic effects. Likewise, if external KCl is varied upward from normal seawater concentration, cellular Na remains remarkably constant. There is no indication of reciprocal changes of internal Na and K as is noted with muscle or nerve. Thus we have no evidence for sodium pumps.

The usual Donnan ratios for Cl and K show no correspondence

except perhaps under the highly abnormal condition of sea water made half molar in KCl.

The general picture is that the head-midpiece fraction is a highly selective ion concentrating mechanism for K in normal sea water. The same system can exclude Na and Cl and, when sufficiently high externally, K as well.

So far as sperm are concerned we would offer the following tentative conclusions:

1. Sperm, as with all other cells studied, concentrate K and exclude Cl and Na as compared to normal sea water or blood.

2. Ion gradient mechanisms depend on activation of head-midpiece portions of sperm. Separated tails have no power to maintain ion gradients.

3. Maintenance of normal conducted flagellar movement depends on ion gradients pumped into the tail by the head-midpiece machinery.

We suggest these findings may have relevance to various aspects of the preservation of sperm and initiation of motility, to be discussed later in this symposium. Sperm collected proximally in the mammalian tract are not motile and are in a high K (low gradient) environment.

On ejaculation, sperm are motile or become motile on exposure to low K-high Na environments which must set up high ion gradients. Isolated sperm tails can still be caused to contract but they do not undulate gracefully in a conducted fashion.

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Ionic and Osmotic Conditions in Relation to Metabolic Control*

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In their natural habitat of the male or female reproductive tract the energy expenditures of mammalian spermatozoa are conserved or expended, inhibited or stimulated, by the local environment. All such controls have undoubtedly evolved as efficient means of assuring performance of ultimate function, fertilization. This process has produced a reproductive cell of amazing versatility and adaptability.

ENERGY EXPENDITURE BY BULL SPERMATOZOA

In general, energy may be expended by spermatozoa which does not result in either motility or the rebuilding of energy-rich adenosine triphosphate (ATP) reserves (the uncoupling of oxidative phosphorylation by 2,4-dinitrophenol (DNP) (Gassner and Hopwood, 1955; Melrose and Terner, 1951, 1952, 1953; Terner, 1957) or the continuance of active oxidation after death of the cells (Kofoed-Johnsen and Mann, 1954; Salisbury and VanDemark, 1957a) are cases in point); but, motility does not occur unless energy is expended. Where the tools available are not adequate to measure their metabolic exchange, the extent and degree of motility by the spermatozoan population *in situ* afford a ready visual certification that metabolic exchange is taking place.

Unfortunately, spermatozoa that have once been actively motile but have lost that capacity owing to some inhibitory treatment may sometimes be considered as dead cells. A dead sperm cell cannot be stimulated to motility. A nonmotile spermatozoon is not always a dead one, but may be an inhibited one without a proper chance to

revive. The nonmotile condition of the living spermatozoa in the mammalian epididymis emphasizes this point, but the fact that immotile sperm cells were not always dead has not been sufficiently recognized in past investigations on sperm motility.

Rebuilding of the limited ATP reserves of mammalian spermatozoa, upon the breakdown of which, with its yield of phosphate-bond energy, the fibrillar contractions depend (Bishop, 1958), may occur under anaerobic conditions only if the cell is supplied with a glycolyzable substrate (Redenz, 1933), fructose, glucose, or mannose, and, in the presence of oxygen, from the oxidation of a variety of substrates. Glycolysis proceeds anaerobically much more rapidly (150 to 250 μg fructose/10^8 cells/hr at 37°C) than it does aerobically (see Table I). By an ingenious use of electrodes inserted into the incubation flask for measurement of impedance-change frequency Walton and Dott (1956) have been able to show that optimum motility of the sperm cell population occurs in the presence of both a glycolyzable substrate and oxygen. Thus, many of our investigations to

TABLE I. Comparative aerobic metabolic activity of bovine spermatozoa from the epididymis, epididymal-like sperm cells (ELC), spermatozoa in semen, and sperm cells washed from semen with and without added fructose at the same comparative concentration of spermatozoa

Type of Spermatozoa	$-Z_{O_2}$ (μl/10^8/hr)	Aerobic $-Z_{fructose}$ (μg/10^8 hr)
Epididymal[a]	3 ± 1.5	
Epididymal, + fructose	8 ± 3	100 ± 20
ELC[b]	4 ± 2	
ELC, + fructose	12 ± 3	60 ± 20
In semen[c]	10 ± 5	40 ± 10
In semen, + fructose	9 ± 5	35 ± 10
Washed sperm cells from semen[d]	6 ± 3[e]	
Washed sperm cells from semen, + fructose	10 ± 5	30 ± 10

[a] Twenty-four samples (Graves et al., 1959).

[b] Sixty samples of sperm cells collected into inhibitory diluents and washed (Graves and Salisbury, 1959).

[c] Several hundred samples, University of Illinois.

[d] Seventeen samples (Lodge and Salisbury, 1960).

[e] This O_2 uptake occurred primarily during the first hour of incubation.

determine the effect of naturally occurring inhibitors and stimulators of spermatozoan metabolic rate have been done in Warburg respirometers under air, with the fructose of semen (or fructose added to washed cells) as the substrate. However, replacement of air by nitrogen is one form of metabolic control. Thus, to obtain ultimate control we have used anaerobic conditions, also.

MODEL CONDITIONS REPLICATED

In assaying the osmotic and ionic requirements for motility of spermatozoa the author and his colleagues have considered the following model conditions with bovine spermatozoa: (1) in the epididymis, where motility and metabolic activity are minimized by lack of substrate, by the balance of cations and anions, and by low gaseous exchange; (2) in semen itself where carbohydrate substrate is plentiful, a change has occurred in the ion balance on ejaculation and the metabolism and motility proceed at intermediate rates under anaerobic conditions but are optimum in the presence of oxygen; and (3) in the presence of the stimulatory secretions of the female upper reproductive tract.

It has not been possible to perform the experiments *in situ*. However, recourse has been made to replication of the media and substrate situation found in the various parts of the male and female reproductive tracts in so far as current knowledge permitted. We have made use of sperm cells in diluted semen, of washed ejaculated sperm cells, and more recently, of specially collected epididymal-like washed spermatozoa. Measurements were made of metabolic activity in the Warburg apparatus by gaseous exchange and by direct chemical analyses. Some interesting observations have been made relative to the control of spermatozoan metabolism and of motility by the conditions employed in these experiments, which are believed to be replication of those found in nature. The primary results will be reviewed here.

RATES OF AEROBIC METABOLISM BY BULL SPERMATOZOA

Table I shows the comparative means and standard deviations of biochemical activity found for bull spermatozoa of different types and for the several conditions tested. The Z (for Zelle) values (mi-

croliters of gas or micrograms of substrate or end product consumed
or produced/10^8 cells/hr) are means of 4-hr incubations in a sus-
pension medium of 0.9% NaCl at 37°C. The first-hour values are
normally higher than the 4-hr means.

It should be noted that the endogenous level of oxygen uptake
by epididymal spermatozoa is low. We have been unable to stimu-
late these cells to increased respiration as suggested by Lardy and
co-workers (1953) except by the addition of utilizable substrate
(Graves et al., 1959). When this is done, however, there is an im-
mediate increase in oxygen uptake. In order to collect routinely,
and at much less expense, a similar cell devoid of substrate, we have
evolved a technique for collection of ejaculated but nonmotile sper-
matozoa directly into inhibitory diluents (Graves and Salisbury,
1959), the basis for which will be clear later in this discussion. These
cells have an aerobic endogenous metabolic rate similar to epididy-
mal spermatozoa and are designated as epididymal-like cells (ELC).
They respond to exogenous fructose by a slightly higher rate of O_2
uptake and a somewhat lower rate of fructose uptake than do epi-
didymal spermatozoa. They respond to all available Meyerhof-
Embden intermediates and to lactate and acetate by sustained mo-
tility and an O_2 uptake similar to that due to fructose but do not
utilize exogenous Krebs-cycle intermediates (Graves and Salisbury,
1960).

Spermatozoa collected as semen are subjected to fructose and
other substrate immediately upon admixture with the secretions of
the accessory glands and until the cells are washed and removed from
them. Because of absorption of substrate during this variable period
before washing, endogenous respiration of washed ejaculated sper-
matozoa is nearly as high at first as in the presence of substrate, but
it decreases in rate as endogenous substrate is exhausted. The re-
sponse to added fructose is variable; an initial depression of respira-
tion often ensues because of the energy yield of glycolysis (Lardy
and Phillips, 1941), but as pyruvate enters, the oxidative scheme
again returns to normal levels (Lodge, 1960).

Figure 1 shows the data of Olds and VanDemark (1957a) on the
oxygen consumption of spermatozoa in semen diluted 1:4 with a
saline solution containing additions from the fluids of the female
reproductive tract in comparison with that in saline alone. This
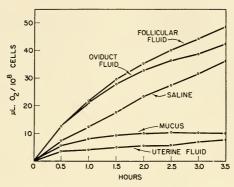
Fig. 1. Effect of ♀ reproductive tract fluids on aerobic metabolism of bull sperm. Number of sperm samples, n, equals 2 (Olds and VanDemark, 1957a).

figure shows clearly that while metabolic activity is depressed by the secretions found in the vagina, cervix, and uterus, it is markedly stimulated by those of the oviducts and follicular fluid.

OSMOTIC PRESSURE AND CATION CONTENT OF FLUIDS FROM MALE AND FEMALE REPRODUCTIVE TRACTS

The osmotic balance and ionic concentration of the primary cations in epididymal fluid (Salisbury and Cragle, 1956), semen (Cragle *et al.*, 1958a, b; Rothschild and Barnes, 1954a, b; Salisbury *et al.*, 1948), and the female reproductive tract fluids (Olds and VanDemark, 1957) are shown in Table II.

Note that the freezing point depression for fluids of the epididymis (Salisbury and Cragle, 1956) is greater than is that of semen (including the spermatozoa) (Salisbury *et al.*, 1948) and that of seminal plasma (Rothschild and Barnes, 1954a). These values are equivalent to a concentration of particles exerting osmotic pressure of about 350–360 milliosmoles in the epididymis, and as low as 285 milliosmoles in seminal plasma. These data, in conjunction with other metabolic data presented later, indicate that one natural means of reducing the metabolic activity of spermatozoa in the epididymis may be its high milliosmole concentration of particles.

TABLE II. Δ F.P., milliosmole concentration and the concentration of bulk cations in male and female reproductive tract fluids and semen of the bovine

| | Δ F.P. °C | Millios-moles | mg/100 ml | | | Na/K ratio |
			Na	K	Ca	
Testis[a]	−0.60	323	111	373	5	0.30
Caput epididymis[a]			235	194	6	1.21
Cauda epididymis[a]	−0.66	355	90	177	5	0.51
Ampulla[a]	−0.62	333	161	240	21	0.67
Seminal vesicle[a]	−0.58	312	250	287	42	0.87
Semen	−0.55[b]	296	271[d]	163[d]	26[a]	1.09
Seminal plasma	−0.53[c]	285	290[d]	161[d]	28[d]	1.80
Sperm cells			173[d]	241[d]	17[d]	0.72
Vagina[e]	−0.65	349	170	166	11	1.02
Uterus[e]	−0.66	353	220	183	15	1.20
Oviduct[e]	−0.65	350	208	223	12	0.93
Follicle[e]	−0.53	287	304	36	12	8.44

[a] Salisbury and Cragle (1956).

[b] Salisbury *et al.* (1948).

[c] Rothschild and Barnes (1954b).

[d] Cragle *et al.* (1958b).

[e] Olds and VanDemark (1957b).

In Table II is shown, also, the concentration of cations (Na, K, and Ca) making up the bulk of the mineral elements in fluids of the male and female reproductive tracts. Also shown is their concentration in bovine spermatozoa expressed for comparative purposes on the same volume basis. It should be noted that the K^+ concentration in these fluids is several times higher than it is in blood. Note the changes in the sodium:potassium ratio from the excurrent ducts of the testes, through the epididymides, the ampullae, semen, and the female reproductive tract. Potassium, in highest concentration in the fluids of the excurrent ducts, is several times higher there than is sodium (Salisbury and Cragle, 1956); whereas the reverse is true, sodium concentration being several times higher, in the follicular fluids (Olds and VanDemark, 1957b). The chloride ion is intimately associated with sodium ion in the male tract fluids, the statistical correlation being about 0.9 (Cragle *et al.*, 1958a). It is nearly as highly correlated with potassium but with opposite sign (Cragle *et al.*, 1958a), reflecting the opposing relationship of sodium and potassium in maintaining osmotic balance (Cragle *et al.*, 1958a; Roth-

schild and Barnes, 1954b). These data, coupled with metabolic data shown in Table I and Fig. 1 and other data to be presented, are interpreted as indicating either that sodium ion has a marked stimulatory effect or that potassium has a marked inhibitory effect on metabolism of mammalian spermatozoa. The latter view seems to fit more satisfactorily the observations (Cragle and Salisbury, 1959). In view of these striking facts we elected, first of all, to study the influence of these bulk cations and some of the anions found in the reproductive tracts as possible mechanisms of natural control of spermatozoan motility and metabolism.

EXPERIMENTAL MODIFICATION OF AEROBIC METABOLISM

Osmotic Pressure

Relatively few experiments uncomplicated by the nature of the ions and other particles exerting the pressure have been conducted to measure the effect of osmotic balance of the suspension medium on the aerobic and anaerobic metabolism of spermatozoa. Several studies (Foote, 1950; Rothschild and Barnes, 1954a, b; Pursley and Herman, 1950; Salisbury *et al.*, 1948) have shown that most of the media first widely used for artificial insemination were hypertonic as compared to bovine seminal plasma. The fact of fertility from their use suggests that the item is not critical. In fact, the studies of Pursley and Herman (1950) indicate that for optimum livability of sperm stored at temperatures above freezing, the freezing point depression of the suspension media may vary from -0.44 to $-0.66°C$ (equivalent to about 235 to 360 milliosmoles).

However, the aerobic metabolism of bull spermatozoa responds differently to variations in osmotic pressure depending on the ions exerting that pressure. The milliosmole concentration of the solutions used in the following experiments has been controlled not only by calculations of the solutes involved but also by measurement of that concentration with a Fiske osmometer. In the first experiment reported here (Cragle and Salisbury, 1959) the solutes were a combination of fructose as substrate, and sodium and potassium citrates and bicarbonates, with the milliosmole concentration varying from 150 to 392 for suspension of washed cells at a final concentration of about 2.0×10^8 cells/ml. The results in Fig. 2 for a 2-hour period
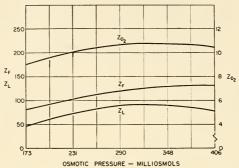
Fig. 2.   Effect of osmotic pressure from combination of Na and K, cit-
rate and bicarbonate, on aerobic metabolism of washed spermatozoa. Num-
ber of sperm samples, n, equals 8 (Cragle and Salisbury, 1959).

at $37°C$ show very little effect of milliosmole concentration on oxy-
gen consumption or aerobic fructolysis.

Response to Varying Osmotic Pressure Caused by Different Cations

For optimum aerobic metabolism in these studies of the effect of
osmotic pressure, we next used 0.9% NaCl as the comparative sus-
pension medium, the salt concentration being varied as indicated
and the dilution ratio being 4 parts of medium to 1 part of semen or
washed cells. The final spermatozoan concentration was controlled
to from 1.5 to 3.5 $\times$ 10^8 cells/ml, and the substrate was seminal
fructose, its degradation products, or added fructose or glucose for
washed cells.

Figure 3 gives the mean oxygen consumption results of one such
experiment with ten semen samples showing the effects of varying
concentrations of NaCl (Lodge and Salisbury, 1960). Both optimum
oxygen consumption and optimum aerobic glycolysis during the 3-
hour period at $37°C$ occurred at a milliosmole concentration isos-
motic with seminal plasma exerting an osmotic pressure of about
280 milliosmoles.

Inhibition by Potassium.   On the other hand, the oxygen uptake
results for parts of the same semen samples suspended 1:4 in a me-
dium composed entirely of KCl at comparable milliosmole concen-
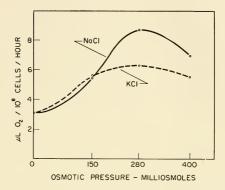
Fig. 3. Effect of osmotic pressure (milliosmole concentration) produced by NaCl or KCl on aerobic metabolism of spermatozoa in semen. Number of sperm samples, n, equals 10 (Lodge and Salisbury, 1960).

tration to the NaCl (Lodge and Salisbury, 1960) are presented in the second curve shown in Fig. 3. These two curves show the difference in metabolic response of spermatozoa to identical milliosmole concentrations of the two major monovalent cations in reproductive tract fluids and semen. The primary effects of osmotic pressure whether exerted by the electrolytes of NaCl or KCl are the same, optimum oxygen consumption and optimum aerobic carbohydrate uptake occurring at an osmotic pressure isotonic with seminal plasma. However, the level of metabolic response in the presence of the Na^+ and K^+ is quite different, the K^+ exerting a marked inhibitory effect on respiration and on lactic acid accumulation, especially at the osmotic pressure exerted by 280 milliosmoles. Fructose uptake was greater in the presence of K^+. While the motility in NaCl was optimum, it was markedly inhibited by KCl, so that at the end of the experiments the spermatozoa in KCl had to be diluted with NaCl to determine that they actually had survived the experiments.

Inhibition by Phosphate. Figure 4 gives the oxygen uptake results of an experiment (Lodge and Salisbury, 1960) with the same ten semen ejaculates in which the effects of the same variations in osmotic pressure are again produced by the monovalent cations, but this time the anion is not the innocuous Cl^-, but the PO_4^{---} at
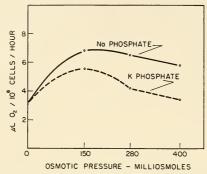
FIG. 4. Effect of osmotic pressure (milliosmole concentration) produced by Na or K phosphate on aerobic metabolism of spermatozoa in semen. Number of semen samples, n, equals 10 (Lodge and Salisbury, 1960).

pH 7.0 which, while required in micro amounts for phosphorylation, is inhibitory of respiration by mammalian spermatozoa when provided in the macro amounts contained in these media (Bishop and Salisbury, 1955; Salisbury and Nakabayashi, 1957; White, 1956). These curves for the 3-hour experiment at 37°C illustrate the fact that the effect of osmotic pressure on the metabolism of mammalian spermatozoa cannot be determined regardless of the specific ions exerting that pressure nor simply by use of one criterion of metabolic activity. Presented in this fashion the curves show that maximum respiration occurred in solutions hypotonic with seminal plasma. The inhibitory effect on respiration is not due strictly to increasing osmotic pressure but rather to the increase in concentration of the phosphate and potassium ions. The experiment shows K^+ to have a simple additive inhibitory effect similar to that shown for K^+ in Fig. 3.

Figure 5 from an earlier report (Salisbury and Nakabayashi, 1957) shows the interactions observed when the cation is Na^+—a comparison of respiratory activity made during 2 hours at 37°C for semen suspended in similarly varying osmotic pressures exerted by NaCl solutions and those composed of sodium dihydrogen and monohydrogen phosphates at pH 7.0. Here the interaction of the nature
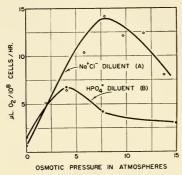
Fig. 5. Interaction of NaCl and NaHPO$_4$ and osmotic pressure on aerobic metabolism. Number of semen samples, n, equals 11 for curve A and 4 for curve B (Salisbury and Nakabayashi, 1957).

of the anion and its concentration as it affected respiration was marked.

Thus a simple statement of the optimum osmotic requirements of mammalian spermatozoa is impossible. To maximize spermatozoan motility and aerobic metabolic activity an osmotic pressure equivalent to a Δ F.P. of fresh seminal plasma (Rothschild and Barnes, 1954a) and of the follicular fluids (Olds and VanDemark, 1957b) ($-0.53°$C) is best. Bishop and Salisbury (1955) several years ago concluded that, for optimum respiration and livability of spermatozoa in semen during 4-hour incubation at $37°$C, dilution with a 0.9% NaCl solution gave better results than other solutions commonly used. Though cells washed in 0.9% NaCl and resuspended in it utilized fructose and motility was sustained, a minimum requirement for K was not then recognized. When excessive washing for removal of substrate was required in some experiments, White (1953) found that minimum levels of K$^+$ were necessary in the washing fluid to maintain subsequent metabolic activity in the presence of substrate. In other experiments Brochart (1951) found that after storage of bull spermatozoa for several days the addition of water or a hypotonic solution to the suspension medium of yolk-citrate restored optimum motility. This last result was most likely due to the

dilution of end products of metabolism which increase on storage resulting in an increased osmotic pressure (Salisbury *et al.*, 1948).

For optimum preservation of spermatozoa and conservation of intracellular reserves the evidence suggests that the proper osmotic pressure is greater than that of fresh seminal plasma, the level in the epididymis being at a milliosmole equivalent of about 350. On the other hand, for optimum resistance to freezing to the temperatures of solid CO_2 or liquid N_2, the glycerol and other constituents in the diluent should exert a Δ F.P. of about $-3.0°$C, equivalent to about a 1.6 molal concentration.

Apart from these considerations is the large number of interactions possible among the variables to which spermatozoa may be naturally and experimentally subjected. Such interacting items include not only osmotic pressure, mineral cations, and the anions already mentioned, but also H ion concentration, other cations, anions, substrates, protein and amino acids, and other constituents of seminal plasma. Most experiments published have dealt with the influence of such factors one, or at most two or three, at a time, so that physiological interactions which might have occurred in the ideal experiment including all such variables at once have not been tested statistically. The published data on the effect of pH on spermatozoan motility, livability, and metabolism illustrate the fact of, if not the degree of, such interactions.

Hydrogen Ion Concentration

While the pH of male and female mammalian reproductive tract fluids upon removal from the tract has been extensively studied in some species, the question needs reexamination *in situ* by modern electronic equipment using microelectrodes so as to eliminate exposure of the fluids to air and thus to rapid gaseous exchange. For example, about twenty years ago Davis and Cole (1939) found that semen of the stallion increased rapidly in pH on exposure to air. Could this have been due to loss of CO_2 from the semen to the atmosphere on ejaculation?

In general, one may say that in the larger mammals the evidence suggests that the pH in the epididymis is low, pH 6.0 or even less in cattle, and that it is increased by admixture of the accessory fluids on ejaculation to near neutrality, depending on the sperm cell con-

centration, it being lower when the cellular concentration is highest (Salisbury and VanDemark, 1961). It is assumed that the buffering capacity of semen will influence the pH of the female tract, which in the cow is more acid in the vagina and uterus (ca. 5.85) than in the oviduct (ca. 6.7) and follicular fluid (ca. 7.3) (Salisbury and Van-Demark, 1961), but this has not been carefully tested in most species.

Literature Shows H Ion Interaction with Other Ions. The effect of pH in the media of suspension on several aspects of ejaculated sperm physiology, including immediate motility (Blackshaw and Emmens, 1951; Emmens, 1947, 1948; Killian, 1933; Moore *et al.*, 1940; Muschat, 1926; Shedlovsky *et al.*, 1942), livability during storage (Anderson, 1947; Emmens, 1947, 1948; Foote, 1950; Lardy and Phillips, 1939; Moore *et al.*, 1940; Romijn, 1948), oxygen consumption (Bishop and Mathews, 1952; Carter, 1932; Lardy and Phillips, 1943; Lardy *et al.*, 1945; Winchester and McKenzie, 1941), fertility and sex ratio of the progeny produced (Casida and Murphree, 1942; Cole *et al.*, 1940; McPhee and Eaton, 1942) in several species including man (Blackshaw and Emmens, 1951; Killian, 1933; Muschat, 1926; Shedlovsky *et al.*, 1942), ram (Blackshaw and Emmens, 1951; Lardy and Phillips, 1939; Lardy *et al.*, 1945; Moore *et al.*, 1940; Winchester and McKenzie, 1941), boar (McPhee and Eaton, 1942; Walton and Dott, 1956), bull, and rabbit (Bishop and Mathews, 1952; Carter, 1932; Casida and Murphree, 1942; Cole *et al.*, 1940; Emmens, 1947, 1948; McPhee and Eaton, 1942), among others have been reported. For bull spermatozoa (Blackshaw and Emmens, 1951; Lardy and Phillips, 1939, 1943; Phillips and Lardy, 1940; Romijn, 1948) the evidence clearly shows that the optimum pH range of the media for some of these functions depends in marked degree on the nature of the ions making up the diluent. For example, some studies show a wide tolerance to pH varying from 5.0 to 8.5 (Bernstein and Beskhlebnov, 1937; Blackshaw and Emmens, 1951; Salisbury and Kinney, 1957) with zones of optimum pH being different for lactate (pH 7.1–8.0), acetate (6.8–8.0), and glycocoll (4.4–5.0) (Bernstein and Beskhlebnov, 1937). Maximum livability at the optimum pH zone varied with the anions in descending order, tartrate, glycocoll, phosphate, lactate, and acetate (Bernstein and Beskhlebnov, 1937). Of these anions both lactate and acetate are

TABLE III. Effect of initial pH of saline-phosphate diluent on respiration and aerobic fructolysis of bovine semen in the presence and absence of metabolic CO_2[a, b]

pH	$-Z_{O_2}$	$-Z_{\text{fructose}}$		$+Z_{\text{lactic acid}}$	
		$-CO_2$	$+CO_2$	$-CO_2$	$+CO_2$
5	7.5	37.7	57.7	12.0	34.3
6	6.7	62.3	82.7	63.3	131.0
7	6.8	74.3	179.3	144.7	223.0
8	8.3	113.7	230.7	203.7	224.7

[a] n, the number of semen samples, equals 12.

[b] Salisbury and Kinney (1957).

rapidly oxidized. Motility ceased at low pH, but the exact pH at which this occurred varied from pH 6.0 for the acetate to pH 4.5 for the phosphate and tartrate buffers. Recovery on raising the pH varied also, being nearly complete in the acetate and least in the phosphate (Bernstein and Baskhlebnov, 1937).

Differential Response of Respiration and Aerobic Glycolysis to pH Variations. Some of our results of the effect of pH on aerobic metabolic activity of bull spermatozoa are shown in Tables III–V and Figs. 6 and 7. In the first of these, Table III, is shown the effect on aerobic metabolism of pH variations of from 5 to 8 (Salisbury and Kinney, 1957). The pH levels were due to differing amounts of sodium monohydrogen and sodium dihydrogen phosphate as one half of a saline-phosphate diluent with a milliosmole concentration of about 290. Respiration was minimal at pH 6 and 7 with a maximum at pH 8. The overall effect was curvilinear with a highly significant pH level by sample interaction; for one or another of the thirty-one semen samples maximum respiration occurred at every one of the pH levels. Note, however, that the effects of pH on aerobic glycolysis were linear, additive, and of substantial quantity per pH unit. Note, also, that both fructose utilization and lactic acid accumulation were highly significantly increased by the presence of the evolved respiratory CO_2 in the reaction flasks.

Figure 6 shows the effect of similar variations in pH in a citrate-bicarbonate buffer on the aerobic metabolism of washed ejaculated spermatozoa of the bull (Cragle and Salisbury, 1959). Again the influence on respiration is curvilinear, but is maximized in the inter-
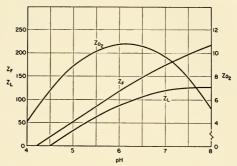
Fig. 6. Effect of pH on respiration and aerobic fructolysis of washed spermatozoa in citrate-bicarbonate buffer. Number of sperm samples, n, equals 8 (Cragle and Salisbury, 1959).

mediate range, about pH 6.0, and is less at the extremes. Aerobic glycolysis is linear and increases with pH, or with decreasing H ion concentration.

Such observations and the published literature suggest the tentative conclusion that oxygen consumption and the spermatozoan enzymes which control it are influenced much more by the other ions than H ion concentration in semen and suspending fluids than is glycolysis and the enzymes controlling it. Glycolysis is, on the other hand, directly and linearly inhibited by H ion concentration below the pH at which glycolysis is optimum. This point has not been clearly established for any diluent, but is clearly at pH 8 or above for the two media illustrated here. Thus, a working hypothesis can be assumed to the effect that, other things being equal, pH of a reproductive tract fluid will reflect the rate of spermatozoan activity in it, both as to motility and energy exchange. In the absence of adequate oxygen, control of metabolic exchange by manipulation of H ion concentration is possible. The body probably does this and the problem for optimum *in vitro* survival is to find optimum conditions for inhibition and reversal of that inhibition.

Combinations of Total Electrolytes, H Ion and K Ion Concentration

Figure 7 shows the results of varying K^+ concentration (at the expense of Na^+) on aerobic metabolism of washed ejaculated bull
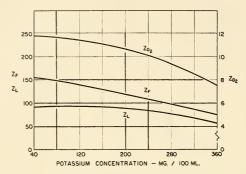
Fig. 7. Effect of K concentration in citrate-bicarbonate buffer on respiration and aeorbic fructolysis by washed ejaculated spermatozoa. Number of sperm samples, n, equals 8 (Cragle and Salisbury, 1959).

sperm cells when the anions are citrate and bicarbonate (Cragle and Salisbury, 1959). Potassium inhibits these metabolic activities independently of the anions with which it is associated as can be seen by comparison of Figs. 3, 4, and 7.

Table IV presents part of the data from a factorially designed experiment testing the manifold effects of osmotic pressure, pH and K^+ concentration in citrate-bicarbonate on washed bull spermatozoa (Cragle and Salisbury, 1959). The data show that oxygen uptake by the cells was not only markedly influenced by K^+ and by pH but by their interaction as well. In fact, the highest and the lowest O_2 uptakes were both at pH 7 and at 231 milliosmoles, but varied inversely

TABLE IV. Effects of osmotic pressure, potassium concentration, and pH on aerobic metabolic activity of washed ejaculated spermatozoa[a, b]

K^+ Conc. mg/ 100 ml	pH	Osmotic Pressure (milliosmoles)									
		348					231				
		$-Z_{O_2}$	$-Z_F$	$+Z_L$	Motility %	Rate	$-Z_{O_2}$	$-Z_F$	$+Z_L$	Motility %	Rate
120	5	11.2	64	4	39	1.7	9.3	46	3	32	1.2
	7	12.4	256	185	44	2.4	12.9	266	179	35	2.1
280	5	8.1	25	0	34	1.0	7.8	+4	0	29	0.8
	7	8.8	221	148	32	1.3	5.0	92	61	17	1.1

[a] n, the number of semen samples, equals 8.
[b] Cragle and Salisbury (1959).

TABLE V. Effects of calcium concentration, potassium concentration, and pH on aerobic metabolic activity of washed ejaculated spermatozoa[a, b]

K+ Conc. mg/ 100 ml	pH	Calcium Concentration (mg/100 ml)									
		12					32				
		$-Z_{O_2}$	$-Z_F$	$+Z_L$	Motility %	Rate	$-Z_{O_2}$	$-Z_F$	$+Z_L$	Motility %	Rate
120	5	6.7	36	43	23	1.1	7.3	40	30	22	1.4
	7	11.4	194	175	30	1.7	14.3	192	170	31	2.4
280	5	6.7	32	39	17	0.9	6.8	12	37	29	1.0
	7	7.2	166	111	21	1.2	9.9	180	134	23	1.7

[a] n, the number of semen samples, equals 8.

[b] Cragle and Salisbury (1959).

with K+ concentration. On the other hand, glycolysis varied directly with pH, inversely with K+, and showed an interaction of osmotic pressure and K+ concentration.

An Interaction with Divalent Calcium

The effect of calcium in a citrate-bicarbonate buffer on aerobic metabolism of washed bull sperm cells is shown in Table V. This cation has presented an enigma. The amount of calcium in the epididymal fluids is no higher than the level of calcium in blood, being less than 10 mg/100 ml. However, three to four times as much calcium is found in fluid in the seminal vesicles which, also, produces the citric acid of semen and, presumably, this would bind the calcium and remove it from chemical reactions. Calcium has been reported to stimulate (Fleig, 1909; Foote, 1950) and to inhibit (Blackshaw, 1953; Lardy and Phillips, 1943) motility (Blackshaw, 1953; Fleig, 1909) and metabolism (Bishop and Salisbury, 1955; Foote, 1950) of spermatozoa. As shown in Table V when combined with variations in pH and K+ in citrate-bicarbonate the calcium ion did not inhibit O_2 uptake or motility. If anything, the higher level of calcium increased respiration and completely removed the inhibitory effect of K+ on glycolysis (Cragle and Salisbury, 1959). These results suggest an important function of calcium in initiating sperm motility at the time of ejaculation. However, in future tests of this point straightforward, single-variable experiments involving calcium will not provide the answers as to its function *in vivo*. That function can be ascertained only if the experiments conducted reflect most of the natural variables involved.

Magnesium

We have not been able to modify the metabolic behavior of either spermatozoa in semen or of washed spermatozoa by normal variations of magnesium in suspension media. Our results suggest that this cation serves primarily as an enzyme catalyst and that even cells washed several times contain enough of it to function properly.

EXPERIMENTAL MODIFICATION OF ANAEROBIC METABOLISM

No one can be quite sure as to the gas exchange conditions existing at all levels of the mammalian male and female reproductive tracts. Bishop has come as close to it as anyone when he found that in the rabbit the pO_2 in the vasa deferentia was too low to support (Bishop and Mathews, 1952), and in the female tubular genitalia (Bishop, 1956) was sufficiently high to support aerobic metabolism. Years ago Redenz (Braus and Redenz, 1924; Redenz, 1925) theorized that the absence of motility by the spermatozoa in the excurrent ducts of the male was due to high pCO_2. This view plus the lack of carbohydrate substrate, the relatively high content of noncarbohydrate reducing substances, the low pO_2, the relatively high osmotic pressure, the high level of potassium, and the low content of calcium in the fluids of the caput and the corpus epididymis are all part of our present working hypothesis. In addition, there is in bull epididymal spermatozoa a diffusible substance (Lardy *et al.*, 1945), containing sulfur, found by Lardy *et al.* (1949), which they have termed a metabolic regulator; in ejaculated semen there is an appreciable amount of sulfite (Larson and Salisbury, 1953).

The following figures and tables will show the present status of our information on some of these points.

Inhibition by Potassium

Figure 8 shows the effect of potassium in Illini variable-temperature diluent (IVT_K) and of sodium (IVT_{Na}) as the primary cation on 4-hour anaerobic glycolysis at $37°C$ of bull semen diluted 1:4 with citrate bicarbonate buffer, as measured by CO_2 evolution from bicarbonate (corrected for both CO_2 retention and acid retention in the buffer) with N_2 as the gas phase (Lodge, 1960). Note again the inhibitory effect of K^+ on glycolysis under these anaerobic condi-
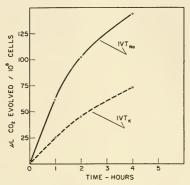
FIG. 8. Anaerobic glycolysis, as measured by CO_2 evolution from citrate-bicarbonate buffer, by spermatozoa in semen under N_2 when the primary cation is sodium or potassium. Number of semen samples, n, equals 3; IVT is Illini variable-temperature diluent (Lodge and Salisbury, 1960).

tions, proving that the K^+ is inhibitory under both aerobic and anaerobic conditions, and possibly by its effect on ATPase as shown in perch sperm flagella by Tibbs (1959).

Similarly, Fig. 9 shows the results from use of the same diluents, IVT_K and IVT_{Na}, on the anaerobic glycolysis of washed spermatozoa,
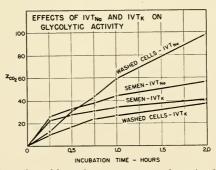
FIG. 9. Effect of washing of spermatozoa and equimolar concentrations of sodium and potassium in citrate-bicarbonate media on anaerobic glycolysis under 5% CO_2, 95% N_2 at 37°C. Number of sperm samples, n, equals 2; IVT is Illini variable-temperature diluent (Salisbury et al., 1960).
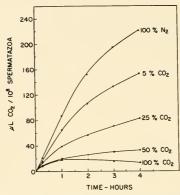
Fig. 10. Effect of pCO_2 on the anaerobic metabolism of bull spermatozoa in semen in IVT_{Na} diluent at $37°C$. Number of sperm samples, n, equals 2; IVT is Illini variable-temperature diluent (Salisbury *et al.*, 1960).

this time under 5% CO_2-95% N_2 (Salisbury *et al.*, 1960). An even more marked effect of K^+ is noted with the washed cells.

Inhibition by pCO_2

Our observations on the effect of varying pCO_2 on anaerobic metabolism of ejaculated bull spermatozoa have been published (Salisbury, 1959; Salisbury and VanDemark, 1957a, b; Salisbury *et al.*, 1960), and the historical development of the idea recently has been reviewed (Salisbury and VanDemark, 1961). Typical results for spermatozoa in semen are shown in Fig. 10 taken from Salisbury *et al.* (1960). The figure shows an increasing inhibition of anaerobic glycolysis with each increase in the pCO_2 to 100%. This is not always the case, however, since sometimes the inhibition is maximized with less than 100% CO_2; this is particularly the case when the diluent contains a high level of K^+.

Effect of pH on Livability under pCO_2 Control

It is well known that the pH of a bicarbonate solution varies with the concentration of CO_2 in the atmosphere above it, and for any given pCO_2 the pH varies directly with the bicarbonate concentration

TABLE VI. Effect of bicarbonate level and of pH on the percentage of motile spermatozoa after storage at 5°C in Na citrate-bicarbonate diluents containing yolk saturated with CO_2 (mean of 11 replications)[a]

Days Storage	Motile Spermatozoa (%)		
Initial	70	71	71
10	60	64	66
20	24	52	60
40	6	29	56
60	0	15	47
Molarity of sodium bicarbonate	0.025	0.05	0.10
Initial pH	6.33	6.53	6.75
Final pH	6.56	6.76	6.98

[a] VanDemark and Bartlett (1960).

of the solution. The pH of a $0.025M$ solution of sodium bicarbonate, first used in our studies, is about 6.2 when saturated with CO_2 gas. This pH is not quite as low as that measured in the fluid of the cauda epididymis of the bull by Branton (6.07 ± 0.13) (Branton, 1946), nor is it low enough to produce maximum control of glycolysis as shown in Table III, IV, or V, and Fig. 6. From these facts and such data as are presented in Table VI we reason that the control of anaerobic glycolysis is not due to the extracellular pH of the diluent. In Table VI the diluent supporting maximum livability of the spermatozoa contained $0.1M$ sodium bicarbonate and was at a pH of 6.75 initially which increased to 6.98 after 60 days' storage. Both of these pH levels would have supported maximum activity. We still know nothing about the intracellular pH of the spermatozoa in the various diluents and pCO_2, but the metabolic control by CO_2 does not result solely from its effect on the pH of the media of sperm cell suspensions.

RELATION OF SULFUR TO SPERMATOZOAN METABOLISM

As stated in the first part of this review, we have been unable to stimulate the endogenous respiration of bull epididymal spermatozoa by additions of sulfide, sulfite, or sulfur-containing organic compounds as reported by Lardy (1953), but have found conditions which remind us of some of the observations he and his co-workers made.

Release of pCO_2 *Inhibition by Sulfite and Its Control by SH*

In Table VII is shown the first of these conditions. Sulfite in amounts comparable to the levels found in semen releases a considerable part of the inhibition of anaerobic glycolysis due to 100% pCO_2 (Salisbury, 1959). This release by sulfite is in turn inhibited by an equivalent sulfur level in the form of sulfhydryl radical of cysteine. Other data on this problem are shown in Table VIII (Lodge and

TABLE VII. Inhibition of anaerobic glycolysis by pCO_2, its partial release by sulfite, and control of the release by sulfhydryl radical, in Na citrate-bicarbonate diluent during incubation of 4 hours at $37°C$[a, b]

	Gas		
	100% N_2	25% CO_2	100% CO_2
	(μl CO_2 evolved by 10^8 cells in 4 hr)		
Control, $1VT_{Na}$[c]	213.5	81.8	2.8
$1VT_{Na}$ + sulfite (30 γ S/ml)	209.0	114.8	79.5
$1VT_{Na}$ + sulfite + cysteine (30 γ S/ml for both)	198.5	79.5	4.8

[a] n, number of semen samples, equals 4.

[b] Lodge and Salisbury (1960).

[c] IVT is the Illini variable-temperature diluent.

TABLE VIII. Inhibition of anaerobic glycolysis by pCO_2, its partial release by sulfite, and effect of free and bound sulfhydryl on the release in K citrate-bicarbonate diluent during incubation of 4 hours at $37°C$[a]

	No. Obs.	Gas			
		100% N_2	25% CO_2	50% CO_2	100% CO_2
		(μl CO_2 evolved/10^8 cells in 4 hr)			
Control, $1VT_K$	57	102.7	21.3	13.1	18.3
+ SO_3[b]	57	82.6	62.1	57.5	66.8
Control	4	95.8	26.1	19.8	27.1
+ SO_3	4	57.0	68.8	62.0	73.4
+ SO_3 + cysteine	4	81.6	35.4	19.5	26.6
Control	4	95.8	37.9	8.7	7.9
+ SO_3	4	86.4	63.9	60.8	60.4
+ SO_3 + methionine	4	79.6	54.1	66.8	61.6

[a] Lodge and Salisbury (1960).

[b] All additions at 30 γ of S/ml.

Salisbury, 1960). The data show for other semen samples that, while the free sulfhydryl radical of cysteine will inhibit the release by sulfite, the bound sulfhydryl radical of methionine will not do so (Lodge and Salisbury, 1960).

Evidence for a Diffusible Metabolic Regulator

The second condition resulted from our need to obtain spermatozoa free from absorbed substrate which had not been stimulated to maximum metabolism and motility. This we have done by collecting semen directly into 100 ml of freshly made CO_2-saturated diluent prepared by mixing citric acid with a potassium and sodium bicarbonate solution (Graves and Salisbury, 1959). With the proper content of K^+ the cells are completely immobilized and do not take up substrate when left in the mixture for 24 hours at $5°C$ (Graves and Salisbury, 1959, 1960). In Fig. 11 is shown the endogenous respira-
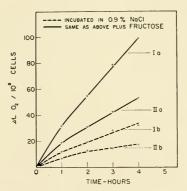
Fig. 11.   Typical oxygen uptake by washed epididymal-like spermatozoa soon after collection with and without substrate, and respiratory stimulation after 24 hours storage at $5°C$ in the inhibitory diluent. I. Collected in 1Na:3K, citrate-bicarbonate CO_2-saturated diluent and stored at $5°C$ for 25 hours before washing and incubation at $37°C$: (a) in saline-fructose (500 mg %); (b) in saline. Number of semen samples, n, equals 12. II. Collected in 0.9% NaCl, and 1Na:1K or 1Na:3K citrate-bicarbonate CO_2-saturated diluent, immediately washed and incubated at $37°C$: (a) in saline-fructose (500 mg %); (b) in saline. Number of semen samples, n, equals 78 (Graves and Salisbury, 1960).

tion of such cells washed and resuspended in 0.9% NaCl immediately after collection (Fig. 11, IIb) and after storage for 24 hours at 5°C in the inhibitory collection diluent (Fig. 11, Ib). Also shown is the respiration of the same cells (Ia and IIa) in the presence of fructose.

The data shown suggest that during the 24-hour storage a change in the cell occurs, perhaps by diffusion of a metabolic regulator from the cells as suggested by the Wisconsin workers (Lardy, 1953; Lardy et al., 1945, 1949), which results in increased respiration both in the absence and presence of exogenous substrate. The observation is entirely in accord with those made on bull epididymal cells by Lardy et al. (1949), and on CO_2-inhibited spermatozoa incubated for 24 hours at 37°C by Salisbury et al. (1960).

EVIDENCE FOR A MINIMUM CO_2 REQUIREMENT

From time to time data have been obtained in our experiments indicating a minimum requirement for free CO_2. One of these observations for aerobic conditions is shown in Table III (Salisbury and Kinney, 1957). Later it was found that optimum oxygen uptake by ejaculated spermatozoa in semen can occur only when CO_2 in amounts normally respired is present (Lodge and Salisbury, 1960). Lodge (1960) also has shown, Fig. 12, that washed epididymal-like
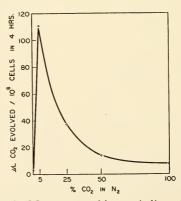
FIG. 12. Effect of pCO_2 on anaerobic metabolism of washed epididymal-like spermatozoa in IVT_{Na} diluent at 37°C. Number of semen samples, n, equal 4; IVT is Illini variable-temperature diluent (Lodge, 1960).

spermatozoa do not glycolyze fructose in a citrate bicarbonate diluent under N_2, but do so when the gas phase contains CO_2. As the pCO_2 is increased above 5%, the typical inhibition of anaerobic glycolysis by CO_2 occurs.

SUMMARY

The mammalian reproductive tract fluids contain many components, some of which exercise the control responsible for the varying degrees of motility and metabolic activity observed for bull spermatozoa at various levels of the male and female reproductive tracts. Some of the components responsible for metabolic control, as judged by their *in vitro* influence on aerobic and anaerobic metabolic behavior of bull spermatozoa, are the presence or absence of substrate; the equivalent molal concentration of the total components; the varying amounts and relative concentrations of the cations making up the bulk of the mineral content of semen, sodium, potassium, and calcium; the concentration of hydrogen ions; the nature of and the relative concentration of some anions; and the partial pressure of oxygen and carbon dioxide. From these studies there emerges evidence of physiological metabolic control of spermatozoa which should lead to a better understanding of one phase of the remarkable reproductive process, and which may be applicable to other tissues as well.

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Oxidative and Biosynthetic Reactions in Spermatozoa*

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At the time the work to be described was started, mammalian sper-matozoa were generally believed to obtain their energy for motility preferentially from glycolytic reactions (Lardy and Phillips, 1941), and a great deal of interest was directed to the study of anaerobic glycolysis, or fructolysis, in spermatozoa of domestic animals (Mann, 1954). There was less concern with the aerobic metabolism of sper-matozoa although the studies of Lardy *et al.* (1945) had produced evidence for the existence of the citric acid cycle in bull spermatozoa.

In order to investigate nonglycolytic pathways, washed bull sperm were incubated with pyruvate in the Warburg apparatus. When an attempt was made to correlate the observed oxygen consumption with the amount of pyruvate metabolized, it was found that much more pyruvate had disappeared than could have been oxidized by the oxygen taken up. Further analysis revealed that about one-half of the pyruvate which disappeared had been reduced to lactate, so that only the remainder, designated "net pyruvate" could have undergone oxi-dation. It was further observed that on addition of 2,4-dinitrophenol (DNP) the formation of lactate from pyruvate was abolished and that the amount of pyruvate oxidized (net pyruvate) was decreased (Table I). Although in this experiment DNP did not alter the rate of respiration, the ratio O_2/net pyruvate was increased to the ex-tent that the pyruvate disappearing could be assumed to undergo complete oxidation. The inhibition by DNP of the metabolism of pyruvate is unusual for animal tissues in which stimulation is usu-ally observed, and also contrasts with the stimulation by DNP of the

TABLE I.   Effect of DNP on metabolism of pyruvate in bull spermatozoa[a]

Additions	$-Z_{O_2}$	$-Z_{pyruvate}$	$Z_{lactate}$	$-Z_{net\ pyruvate}$
None	18.1	—	—	—
Pyruvate	25.9	45.6	24.0	21.6
Pyruvate, DNP	25.8	8.0	0.6	7.4

[a] Washed spermatozoa incubated in phosphate saline at $37°$; gas, air; pyruvate, $0.01M$; DNP, $10^{-4}M$; incubation period 45 min; sperm count 9.7×10^8/vessel.

aerobic breakdown of carbohydrate in spermatozoa. In bull spermatozoa incubated with glucose or fructose, the addition of DNP results, as in other animal tissues, in stimulation of the rate of respiration and also of the rate of accumulation of lactic acid under aerobic conditions (Melrose and Terner, 1953).

At a later stage the adoption of a more convenient technique dispensed with the tedious analytical determinations. By using C^{14}-labeled substrates, the respiratory CO_2 can be collected, and its radioactivity provides a measure of the amount of substrate oxidized. Table II shows that of glucose, acetate, and pyruvate, the last was oxidized most readily, which would not have been apparent from measurements of the oxygen uptake alone. Also the different types of responses to DNP of spermatozoa metabolizing pyruvate or carbohydrate are shown. The formation of lactate from pyruvate by

TABLE II.   Metabolism of acetate, pyruvate and glucose in bull spermatozoa[a]

Additions	$-Z_{O_2}$	Labeled Substrate Appearing in Respiratory CO_2 ($\mu l/10^8$ spermatozoa/hr)
None	9.6	—
DNP	5.8	—
1-C^{14}-acetate	28.5	8.9
1-C^{14}-acetate, DNP	32.0	11.3
3-C^{14}-pyruvate	30.2	14.7
3-C^{14}-pyruvate, DNP	21.0	5.3
C^{14}-glucose	23.0	4.6
C^{14}-glucose, DNP	40.9	8.0

[a] Washed spermatozoa incubated in phosphate saline at $37°$; gas, air; 1-C^{14}-acetate, $0.01M$; 3-C^{14}-pyruvate, $0.01M$; C^{14}-glucose (r.l.), $0.005M$; DNP, $10^{-4}M$; incubation period, 90 min; sperm count, 9.42×10^8/vessel.

TABLE III. Effect of DNP on anaerobic metabolism of pyruvate in bull spermatozoa[a]

Additions	$C^{14}O_2$ ($\mu l/10^8$ spermatozoa/hr)
None	9.9
DNP	4.6
Fluoride	9.9
Fluoride, DNP	4.2

[a] Washed spermatozoa incubated in phosphate saline at $37°$; gas, 100% N_2. 1-C^{14}-pyruvate, $0.005M$ in all vessels; DNP, $10^{-4}M$; fluoride, $0.02M$; incubation period, 60 min; sperm count, 19.89×10^8/vessel.

washed spermatozoa led to the conclusion that bull spermatozoa contain a powerful enzyme system which catalyzes the dismutation between two molecules of pyruvate, analogous to the reaction studied in bacterial extracts by Korkes *et al.* (1951):

$$2CH_3CO\ COOH + CoASH \rightarrow$$

$$CH_3CO\ S\ CoA + CH_3CHOH\ COOH + CO_2$$

This reaction would account for the reduction of pyruvate to lactate in the absence of carbohydrate under both aerobic and anaerobic conditions. It can be followed by measuring the amount of radioactive CO_2 evolved from 1-C^{14}-pyruvate by a sperm suspension incubated under strictly anaerobic conditions. As can be seen from Table III, the anaerobic decarboxylation of pyruvate is also inhibited by DNP (Terner, 1959).

What is the significance of the dismutation reaction of pyruvate in bull spermatozoa? It might be argued that a study of the metabolism of pyruvate is only of academic interest since the physiological substrate is carbohydrate and, in particular, fructose. And it is generally assumed that bull spermatozoa can obtain all the energy for the maintenance of motility from anaerobic glycolysis. That would make a study of the fate of pyruvate, other than its reduction to lactate in the course of glycolysis, relatively unimportant. For it may be expected that in the presence of carbohydrate the dismutation between two molecules of pyruvate will be suppressed, giving way to the better known and more important oxidation-reduction step of glycolysis.

TABLE IV. Effect of anaerobic glycolysis on decarboxylation of pyruvate[a]

Additions	$C^{14}O_2$ ($\mu l/10^8$ spermatozoa/hr)
None	8.3
DNP	2.8
Glucose	4.1
Glucose, DNP	0.6
Fluoride	8.7
Fluoride, DNP	3.1
Glucose, fluoride	5.9
Glucose, fluoride, DNP	1.3

[a] Washed spermatozoa incubated in phosphate saline at 37°; gas, 100% nitrogen; 1-C^{14}-pyruvate (0.005M) in all vessels; glucose, 0.005M; fluoride, 0.01M; DNP, $10^{-4}M$; incubation period, 75 min; sperm count, 9.1×10^8/vessel.

Before making this almost obvious conclusion, it was tested experimentally. Contrary to expectation, it was found that the decarboxylation of 1-C^{14}-pyruvate persisted in the presence of glucose. When fluoride was added to minimize isotope dilution of the labeled pyruvate by unlabeled pyruvate produced by glycolysis, there was only 30% suppression of the decarboxylation of pyruvate (Table IV). It seems that the dismutation system is powerful enough to compete with the glyceraldehyde phosphate dehydrogenase system. And it appears that even under anaerobic conditions, a part of the pyruvate derived from glycolytic breakdown of fructose undergoes dismutation producing lactate, CO_2, and acetyl coenzyme A. The production of acetyl coenzyme A under anaerobic conditions raises the question of its utilization. In the absence of oxygen the acetyl coenzyme A cannot be oxidized via the Krebs cycle. It may be simply deacylated to acetate and coenzyme A, but a more interesting possibility is that acetyl coenzyme A serves as the starting point of biosynthetic reactions in spermatozoa under both aerobic and anaerobic conditions.

Biosynthesis in spermatozoa had been kept in mind for some time, ever since the assumption was made, rightly or wrongly, that the incomplete oxidation of pyruvate pointed in this direction (Melrose and Terner, 1953). An attempt was made to investigate biosynthetic reactions in bull spermatozoa, but before these results are described,

TABLE V.   Oxidation of glucose, pyruvate, and acetate by spermatozoa[a]

Count/flask $\times 10^8$	Bull 9.42		Man 0.38		Sucker 117.1		Trout 315.4	
Substrate	O_2	$C^{14}O_2$	O_2	$C^{14}O_2$	O_2	$C^{14}O_2$	O_2	$C^{14}O_2$
C^{14}-glucose (r.l.)	2300	460	?	33.6	118	0.32	21.3	0.22
2-C^{14}-pyruvate	3020	1470	?	26.4	138	21.20	22.2	2.57
1-C^{14}-acetate	2850	890	?	16.4	110	8.80	19.7	1.54
None	960	—	?	—	120	—	20.0	—

[a] Metabolic rate ($\mu l/10^{10}$ spermatozoa/hr)

a few words will be said about some measurements of the oxidative activity of spermatozoa of other species.

Table V lists determinations of the rates of oxidation of glucose, pyruvate, and acetate in spermatozoa of the bull, man, and fish. The main difficulty in working with human semen is its small volume and its low sperm count. Its average volume is 3.5 ml, and its count is of the order of 100 million cells/ml. Bull semen has a count ten times as high and larger volumes are usually available. Fish sperm can be obtained in large amounts, but the spawning season is relatively short.

The sperm suspensions were centrifuged and resuspended in Krebs saline. The respiration was measured in the Warburg apparatus, but no readings were obtained in the case of human spermatozoa. Nevertheless, by the more sensitive isotope technique it could be shown that human spermatozoa oxidized glucose, acetate, and pyruvate to CO_2 and water. This seems to be unequivocal evidence that human spermatozoa have an aerobic metabolism (Terner, 1960).

The metabolic rate of bull spermatozoa was by far the highest of those listed here. Although it is customary to use the Z notation (microliters per 10^8 spermatozoa per hour), the metabolic rates are in Table V given as microliters per 10^{10} spermatozoa per hour.

The oxidation rates in human and fish spermatozoa are of a similar order of magnitude, whereas the metabolic activity of bull spermatozoa is much greater. In bull and fish spermatozoa, the rate of oxidation of pyruvate stands out as the highest, but whereas the mammalian spermatozoa oxidize glucose quite well, fish spermatozoa

hardly oxidize it at all. Of the two species of fish shown here, the sucker produced spermatozoa of good motility, whereas the spermatozoa of the trout (brown and rainbow trout) were of poor motility, and the metabolic rates were much lower, although the pattern is the same.

The question whether human spermatozoa have a respiratory metabolism has long been in doubt. It should be obvious from the data why the oxygen consumption of isolated human spermatozoa has perhaps never really been measured. Fortunately the sucker is related to man at least in the rate of substrate oxidation by spermatozoa. If it is assumed that human spermatozoa have an endogenous respiration as high as sucker sperm (which is unlikely), the oxygen uptake of human sperm could not exceed $100 \ \mu l / 10^{10}$ spermatozoa/hr. This gives a maximum Z_{O_2} of $ca. \ -1$, that is, the oxygen uptake would be $1 \ \mu l / 10^8$ spermatozoa and probably much less than that. Since 10^8 spermatozoa is the average content of 1 ml of human semen, not much can be expected from manometric measurements even using micro-Warburg vessels.

Another question regarding the metabolism of human spermatozoa is whether their oxidative capacity is not so low that it may be dismissed as insignificant in comparison with the glycolytic system. Here comparative studies may be useful. Sucker spermatozoa oxidize pyruvate at a rate similar to that of human spermatozoa and utilize hardly any glucose. In fish spermatozoa, respiration has long been known to be of far greater importance for the support of motility than glycolysis (see Mann, 1954), and our isotope experiments confirm that glycolysis is relatively slow. If oxidations at this "low" rate are adequate to sustain fish sperm, they may also be useful to human spermatozoa. The point that is made is simply this: if the supply of fish sperm were as scanty in volume and sperm density as the single human ejaculates on which manometric measurements have so far been conducted, the conclusion might well be that fish spermatozoa lack both the glycolytic and the oxidative mechanisms of energy production. It is interesting to speculate what other mechanisms imagination might have led us to propose as an alternative.

It seems odd that man is closer to fish than to the bull as far as the rate of sperm metabolism is concerned. The spermatozoa of mammals, on the other hand, have in common the possession of a power-

ful glycolytic system, which is lacking in fish sperm. From the evo-
lutionary point of view, glycolysis is believed to be the more primi-
tive mechanism. But there is no reason why this should be so in the
case of spermatozoa which are produced at an evolutionary stage
at which respiration is already firmly established. It is possible that
the maintenance of motility of spermatozoa of certain lower forms
depends on oxidative metabolism and that the development of sper-
matozoa capable of deriving energy from glycolytic processes is a
later development in mammals.

Now to return to studies of biosynthetic reactions in bull sperma-
tozoa: These were washed free from seminal plasma and incubated
with C^{14}-labeled glucose, glycerol, or acetate on as large a scale as
possible. It took several months to accumulate enough material for
each run. The cells were extracted with lipid solvents, and the ex-
tracts were fractionated by silicic acid column and paper chroma-
tography. Thus far there have been isolated a diglyceride fraction
of high specific activity, less strongly labeled triglyceride and phos-
phatide fractions, four weakly radioactive zones which appear to be
ketosteroids, and cholesterol, which contained very little, if any, C^{14}
label. The diglycerides, cholesterol, and ketosteroids were eluted
from the silicic acid column in one strongly radioactive fraction (25%
ether/75% hexane). They were further fractionated by paper chro-
matography in the Zaffaroni system. This treatment left several zones
on the paper which gave a positive Zimmerman reaction. The fastest
moving zone was allowed to run off, and it contained cholesterol
and glycerides. The cholesterol was removed by treatment with
digitonin and the digitonin-soluble supernatant was again purified
on a silicic acid column. This yielded a relatively homogeneous frac-
tion which was identified as consisting of diglycerides by its running
properties on the column and on paper (Schlenk *et al.*, 1957), and by
infrared spectrophotometry which showed the progression curve
characteristic of long-chain fatty acids. The diglyceride fraction
which was strongly C^{14}-labeled was transesterified, and the fatty acid
methyl esters were analyzed by gas chromatography. The fatty acids
found were mainly myristic, palmitic, and stearic acids, but in some
samples oleic acid and lauric acid were also present. The C^{14} label
was not in the fatty acids but in the glycerol moiety, when the incu-
bation substrate had been C^{14}-glucose or C^{14}-glycerol. The glycerides

were only weakly labeled when $1\text{-}C^{14}$-acetate, in the presence or absence of unlabeled glycerol, was the radioactive substrate. In addition, the labeling of the glycerol of the diglycerides occurred in bull spermatozoa incubated with C^{14}-glucose under both aerobic and anaerobic conditions (Terner and Korsh, 1960, 1962).

Although the cholesterol was almost free from radioactivity, the ketosteroid fraction was radioactive. The latter (it had been separated from a strongly radioactive mixture) was purified by repeated paper chromatography in the Zaffaroni and Bush systems. Four radioactive zones were detected by radioautography, and each of these gave a positive Zimmerman reaction.

The phosphatide fractions recovered from the silicic acid columns were run on silicic acid impregnated paper (Marinetti *et al.*, 1957). These were of much lower specific activity than the glycerides, but they also became labeled under anaerobic as well as aerobic conditions. At least one component was obtained in a state sufficiently pure for identification by infrared spectrophotometry. It showed the bands characteristic of lecithin (Freeman *et al.*, 1957). It had been necessary to store the samples for long periods of time in order to accumulate sufficient material, and there was much degradation as shown by the appearance of lysolecithins. Therefore, no attempt was made to isolate plasmalogens which have been reported to be present in fresh spermatozoa (Lovern *et al.*, 1957).

It is known that diglycerides are precursors of triglycerides and phosphatides (Kennedy, 1957), and the relative specific activities of the compounds isolated from the bull spermatozoa incubated with radioactive substrates are in agreement with this order of events.

On the question of the substrate of endogenous respiration Hartree and Mann (1959) have suggested that the fatty acids of the acetalphosphatides present in ram and bull spermatozoa are the substrates. Our studies show a rapid incorporation of glycerol into diglycerides and therefore suggest a rapid turnover of these compounds. This makes it very probable that the glycerides constitute a readily available source of substrate for the maintenance of the endogenous respiration. In fact the amounts of diglyceride isolated were more than sufficient to support respiration at a rate corresponding to a Z_{O_2} of -10 to -20 for one hour.

A reappraisal of the role of aerobic and anaerobic glycolysis in

bull spermatozoa is now possible. That the breakdown of carbohydrate by spermatozoa results in the production of ATP for motility and also has a sparing effect on endogenous lipids because of the preferential oxidation of carbohydrate has long been recognized. Most sperm physiologists will readily agree that the breakdown of carbohydrate by spermatozoa not only results in the production of ATP but also has a sparing action on the endogenous lipid reserves owing to the preferential oxidation of carbohydrate. Beyond that, however, there is a long-standing belief, evident also in the present symposium, that the energy generated by glycolysis is utilized exclusively in the support of motility, disregarding and even discounting the possibility of the utilization of ATP in reactions resulting in the maintenance of some of the constituents of spermatozoa. The work that has been described shows that the effect of carbohydrate in maintaining endogenous lipid reserves is more than a sparing of the lipid. Rather it is due to an active synthesis of the lipids since we have seen that carbohydrate carbon appears in the glycerol moiety of the lipids of isolated spermatozoa on incubation under both aerobic and anaerobic conditions.

It has also been found that there is, in bull spermatozoa at least, a mechanism by which acetyl coenzyme A may be formed from carbohydrate even in the absence of oxygen and that this may be utilized in fatty acid biosynthesis.

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Respiration and Oxidative Phosphorylation in Relation to Sperm Motility

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Few isolated cells are as intriguing as the spermatozoon. By its vital function, peculiar anatomy, capacity for motility, limited ability for independent survival, this cell, derived from body tissues, appeals greatly to the biological chemist.

The investigator would like to know to what extent this specialization is paralleled by a modification in metabolism; whether the same enzymes, in analogous proportions or concentrations and with comparable activities, are present in both the tissues and spermatozoa of a given animal. Respiratory enzymes appear particularly well suited for such a study when one bears in mind the classical difference between spermatozoa of mammals and those of marine invertebrates. The latter rely for their activity on oxidative phosphorylation, a pattern fifteen times more favorable in terms of energy yield than the glycolytic pathway preferentially adopted by many mammalian spermatozoa. This difference has often been compared with the contrasting ecological conditions accompanying external and internal fertilization. The spermatozoa from marine animals, impermeable to or unable to utilize sugars and thus relying on internal metabolites, are released into a non-nutrient aerobic medium, sea water, whereas, the spermatozoa of mammals may be found in an environment rich in substrates, but possibly anaerobic. The significance of this situation needs clarification especially in view of the fact that low oxygen tension apparently can support a normal rate of respiration in mammalian spermatozoa.

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Certain explanations for this striking difference in cells from different groups of animals but otherwise of the same type will be considered below. It can be seen that if their respective equipment in oxidative enzymes is very much alike, the difference must appear at the level of energy conservation, namely in the efficiency of the coupling of phosphorylation to oxidation.

The data are presented in three parts. First, the results are given of a qualitative survey of cytochromes and other respiratory enzymes in the spermatozoa of the dog, bull, and marine mollusk, *Spisula*. In a second section, the proportions, concentration, and turnover numbers of enzymes are tabulated, and comparisons are made among spermatozoa and between these and body cells of the parent organism. Finally, the data pertaining to enzymic activity in relation to certain substrates and specific inhibitors are presented and correlated with motility of the cells.

EXPERIMENTAL PROCEDURES

Biological Material

Freshly collected bull semen, diluted about five times with skimmed milk, was received weekly from a nearby artificial breeding cooperative (Southeastern Pennsylvania Artificial Breeder's Cooperative). During transportation and prior to use, the samples were maintained at $10°C$. The same three donor bulls were used throughout (H 43, H 47, H 51). Spermatozoa numbered 50 to 200×10^8 per ejaculate, and the motility ratings given by the Cooperative were 50 to 80%. For spectrophotometric experiments the sperm suspensions were washed three times, at $20°C$, with a modified Ringer of pH 7.2 (NaCl $0.13M$, KCl $0.006M$, $MgSO_4$ $0.002M$, Na_2HPO_4 $0.016M$) keeping the original dilution so far as possible. Centrifugations were run at 1000 rpm for 20 minutes. The final working dilution was 5 to 10×10^8 cells/ml as estimated by turbidimetric and microscopic methods. Fair motility was retained for a few hours in the saline medium supplemented with glucose.

Dog semen was obtained at the University of Pennsylvania Medical School and studied immediately after collection. The number of spermatozoa per ejaculate was only 5 to 10×10^8; therefore, semen from five dogs had to be pooled and concentrated by one or two

centrifugations to attain the required final cell concentration of 5×10^8/ml. The spermatozoa were suspended in the above modified Ringer, in seminal fluid, or in mixtures of both, buffered at pH 7.4 by $0.02M$ glycylglycine. Motility was high in the presence of glucose or seminal fluid.

Spisula spermatozoa were collected from the punctured gonads of ripe animals. The sperm suspension was passed through cheesecloth, washed once, centrifuged at 3000 rpm, and finally resuspended in sea water buffered by $0.02M$ glycylglycine at pH 7.5. Final cell concentration was 10 to 30×10^8 cells/ml. Motility was high in the samples studied. These studies on clam sperm were performed at the Marine Biological Laboratory (Woods Hole) and the Johnson Foundation (University of Pennsylvania).

Methods

Absorption spectra at room temperature were obtained with the wavelength-scanning, recording spectrophotometer described by Yang and Legallais (1954). Spectra at the temperature of liquid nitrogen, run in the presence of glycerol (Keilin and Hartree, 1949), were recorded by the procedure of Estabrook (1958). At liquid nitrogen temperature, difference spectra, without addition of glycerol, were also obtained on "stabilized steady states" according to the method of Chance and Spencer (1959). Oxygen consumption was determined on aliquots before and after spectroscopic measurements. The recording platinum and silver electrodes were dipped in a rotating cuvette (0.5 to 2 ml) and polarized at -600 mv (Davies and Brink, 1942; Williams, 1961). Motility was estimated by microscopic examination of pairs of drops pipetted from treated and reference samples. In later stages of the work an apparatus modified from that of Bosselaar and Spronk (1952) and of Rikmenspoel (1957) was used which gave relative motility ratings under controlled conditions and at the same time registered pH changes and oxygen concentration (Gonse, 1960).

Calculations

The concentrations of respiratory enzymes may be obtained from optical density changes at selected pairs of wavelength on difference spectra at 25°C. One applies the molecular extinction coefficients

given by Chance (1954a, 1957) and Chance and Williams (1955), namely, 16, 19, and 22 for the alpha absorption bands of cytochromes a, c, or c_1, and b, respectively. The coefficient 22 was also considered valid for the b-like cytochromes of *Spisula*. A coefficient of 11 at the trough of reduced flavoproteins at 460 mμ, of 6 for pyridine nucleotides at 340 mμ, and of 91 for cytochrome a_3 at the Soret absorption band were also adopted.

Presentation of Results

The experimental procedure to be followed in recording a series of difference spectra on a single preparation has been described by Chance (1954b). Briefly, an experiment at room temperature begins with a pair of cuvettes (2 ml) filled with the same suspension which, on recording, yields a flat base line. Modification of conditions alternatively in each cuvette gives, step by step, difference spectra and base lines. Spectra are described here (e.g., Fig. 1) by giving, inside the first parentheses the successive treatments performed on the more reduced sample, followed by a minus sign and a second parenthesis summarizing successive conditions performed on the more oxidized sample.

Abbreviations used in the text and figures are: RP for Ringer phosphate medium, GG for glycylglycine, SW for sea water, PN for pyridine nucleotides, FP for flavoproteins, DNP for 2,4-dinitrophenol, and TN for turnover number. The notation Z_{O_2} designates oxygen consumption of 10^8 spermatozoa expressed in microliters of O_2 per hour.

RESULTS

Identification of Respiratory Enzymes.

A rather complete picture of the metabolically reducible respiratory pigments is obtained from a recording of the spectroscopic difference between the washed starved aerobic sperm sample and the anaerobically reduced one. The starved sample of bull spermatozoa (Fig. 1) has a low respiration on the order of 0.01 μM O_2/sec (Z_{O_2} of about 1) and remains aerobic for the duration of the experiments, whereas in the other sample, addition of 20 mM lactate evokes a tenfold increase of respiration and exhaustion of oxygen in 5 minutes.
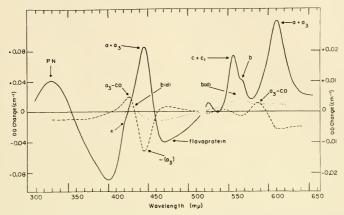
Fig. 1. Difference spectra of bull spermatozoa at 25°C. Cells washed and suspended in RP, pH 7.2, 8.9 × 10⁸ cells/ml. Sample H477. Solid line: (anaerobic, lactate 20 mM) − (aerobic, endogenous substrates). Dashed line: (anaerobic, lactate, CO) − (anaerobic, lactate). Dotted line: (anaerobic, lactate, CO, dithionite) − (anaerobic, lactate, CO).

Before this point is reached, a partial reduction corresponding to the aerobic steady state of lactic acid metabolism can be recorded, as will be seen later. Also shown (Fig. 1) is the formation of a cytochrome a_3-carbon monoxide complex with absorption bands at 585 and 425 mμ. The disappearance of the cytochrome a_3 absorption which follows the treatment by CO (trough at 445 mμ) and comparison between this trough and the previous Soret band at the same wavelength indicate a one to one ratio of cytochrome a_3 to cytochrome a. The effect of dithionite on anaerobically reduced samples shows the presence of a chemically reducible component of the cytochrome b type, called $b(d)$.

On a number of spectra, obtained at room temperature with various differential arrangements, absorption bands have been located at the following wavelengths: at 605 and 445 mμ absorption bands corresponding to reduced cytochromes a and a_3, and at 552 mμ and 415 mμ bands indicative of cytochrome c. The 552 mμ absorption is in fact due to the mixture of cytochromes c and c_1. After one-hour extraction by distilled water, which removes cytochrome c, cyto-
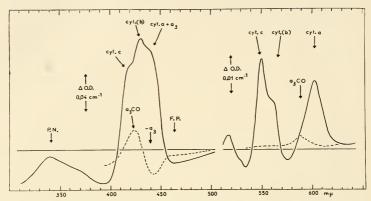
Fig. 2. Difference spectra of *Spisula* spermatozoa at 25°C. Cells in SW-GG, pH 7.3, 37 × 10⁸ cells/ml. Sample S9. Solid line: (anaerobic) − (aerobic, Amytal 10 mM). Dashed line: (anaerobic, CO) − (anaerobic).

chrome c_1 absorption stands out at 554 mμ. Cytochrome b absorption bands are found at 562 mμ and 430 mμ, while cytochrome $b(d)$ absorbs at 564 mμ. A trough at 460 mμ is indicative of flavoproteins, and absorption at 340 mμ of pyridine nucleotide, both in the reduced state. The location of absorption bands has been checked and corrected with reference to a sample of pure beef heart cytochrome c.

Mann (1945, 1951) demonstrated the presence of cytochromes a_3, a, b, and c in spermatozoa of mammals and pointed out the greater abundance of cytochrome oxidase. This feature is particularly striking if a comparison is made of the spectra of bull (Fig. 1) and *Spisula* spermatozoa (Fig. 2) obtained also under anaerobic conditions. In *Spisula* sperm, owing to the more common distribution of cytochromes a and a_3, the gamma bands of cytochromes c and (b) remain distinct instead of fusing into the single peak at 445 mμ as is characteristic of bull sperm.

In *Spisula* spermatozoa, at 25°C, absorption bands at 603 and 440 mμ indicate the presence of cytochromes a and a_3, the latter band being shifted toward shorter wavelength by cytochrome (b) absorption at 430 mμ (Fig. 2). After treatment with carbon monoxide, an a_3-CO compound is formed with absorption at 590 and 425 mμ. Cyto-
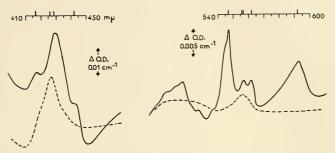
Fig. 3.   Difference spectra of *Spisula* spermatozoa at liquid nitrogen temperature. Cells in SW-GG. Sample S10. Solid line: (anaerobic, KCN 8 mM) − (aerobic, endogenous substrates). Dashed line: (anaerobic, KCN, dithionite) − (anaerobic, KCN).

chrome c is evidenced by absorption bands at 550 mμ and 417 mμ. A cytochrome b-like component absorbs at 562 and 430 mμ. But at low temperature, spectrophotometric records indicate that this (b) component is actually double with alpha absorption bands at 557 and 562 mμ (Fig. 3). If a shift of 3 mμ toward shorter wavelength is assumed upon cooling, these pigments, at $25°$C, should absorb at 560 and 565 mμ; however, what is actually found is a fused absorption at 562 mμ, here attributed to cytochrome (b).

Among other features of Fig. 3, there is a noticeable shift of the alpha absorption of cytochrome a in the presence of KCN, which, as well as azide, under aerobic conditions brings about reduction of terminal cytochromes. With addition of dithionite in an anaerobic sample, absorption bands appear at 556 and 427 mμ and indicate further reduction of a (b) component, possibly the lower of the (b) cytochromes previously defined.

As noted for *Spisula* sperm low-temperature spectroscopy permits separation of nearby absorption bands. In dog or bull spermatozoa (Fig. 4) one can distinguish in this way, without separate specific treatments, cytochromes a_3, a, c, c_1, b, and $b(d)$ by their absorbencies at 444, 601, 548, 553, 558, and 561 mμ, respectively. Cytochrome $b(d)$ is particularly noticeable at low temperature by its beta absorption at 535 mμ. Attention is directed to the similarity between spectra obtained on dog and bull spermatozoa.
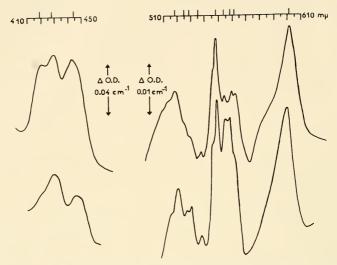
Fig. 4.   Difference spectra at liquid nitrogen temperature in the presence of 50% glycerol. Samples H476 and M5. (Dithionite) − (aerobic, endogenous substrates). Upper curves: bull spermatozoa, washed and suspended in RP, pH 7.2. Lower curves: dog spermatozoa, lightly washed and suspended in RP-GG, pH 7.3.

Following extraction of cytochrome c from bull spermatozoa, the absorption band of cytochrome c_1 stands out clearly at 552 mμ at low temperature (Fig. 5). On the same material, difference spectra also recorded at liquid nitrogen temperature indicate dithionite-reduced cytochrome $b(d)$ at 561 mμ; in dog sperm after freezing and thawing, a dithionite-reducible material, analogous to mitochrome and distinct from cytochrome $b(d)$, absorbs at 555 mμ (Fig. 5). The existence of a cytochrome with absorption at 561 mμ, instead of 558 mμ as for cytochrome b (at low temperature), is further demonstrated in Fig. 6. In this spectrum of dog spermatozoa, cytochrome $b(d)$ appears at 560.5 mμ, distinctly separated from cytochrome b (558 mμ), but in this case reduction follows treatment with the respiratory inhibitor, antimycin A, instead of dithionite. Cytochrome $b(d)$ is repeatedly found
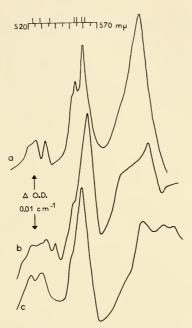
FIG. 5. Spectra at liquid nitrogen temperature in the presence of 50% glycerol. Samples H434, H437, M1. (a) Bull spermatozoa, suspended in RP after 4 days extraction by RP at 5°C; absolute spectrum. (b) Bull spermatozoa, washed and suspended in RP, (anaerobic, KCN 1 mM, dithionite) — (anaerobic, KCN 1 mM). (c) Dog spermatozoa, suspended in RP-GG; frozen and thawed, refrozen for recording, both cuvettes treated the same way, one supplemented with dithionite for difference spectrum.

in spermatozoa of dog and bull and therefore appears to be a normal component rather than an artifact due to cell alteration as is the case after freezing and thawing. The metabolic role of this component is not as yet clear. It is not reducible by anaerobiosis or by oxidizable substrates under aerobic conditions; it is antimycin-sensitive. Chance (1958) distinguishes also three (b) cytochromes in
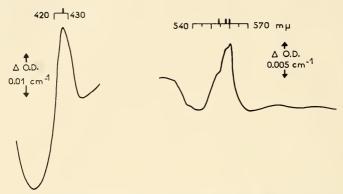
Fig. 6.   Difference spectrum of dog spermatozoa at liquid nitrogen temperature. Cells washed and suspended in RP-GG, pH 7.3. Sample M6. (Antimycin A $1.5 \times 10^{-5}M$, lactate 10 mM) — (lactate 10 mM); both aerobic.

succinic oxidase preparations with somewhat analogous properties to those found here and absorption bands at 566, 560, and 556 mμ (room temperature).

In bull spermatozoa we have thus demonstrated the same respiratory pigments as those in body tissues: cytochromes a_3, a, c, c_1, b, flavoproteins, and pyridine nucleotides. At the more detailed level, distinctions can be drawn between true cytochrome b, antimycin-sensitive cytochrome b, and mitochrome.

Few data are available on the respiratory pigments of *Spisula* tissues. A report by Kawai (1959) described cytochromes a_3, a, and c in lamellibranchs, whereas only one (b) cytochrome was found which was considered as true cytochrome b. The technique used (room temperature) did not permit detection of close components. According to Bonner (1961) muscle tissues of scallops and *Spisula* show, at low temperature, the presence of distinct 557 and 560 mμ cytochromes (b) both of which are antimycin-sensitive. It seems, therefore, that little if any difference is present in the set of pigments in the tissues and spermatozoa of *Spisula*: cytochromes a_3 and a, cytochrome c, cytochrome (b) (560 mμ at low temperature), cytochrome (b) (557 mμ at low temperature and possibly identical with true cytochrome b), fla-

voproteins, and pyridine nucleotides. No cytochrome c_1, as found in sea urchins (Keilin and Hartree, 1949), was detected.

Concentration of Respiratory Enzymes

From a number of spectra such as those shown in Figs. 1 and 2, recorded at room temperature, one can calculate proportions and concentrations of the respiratory enzymes. Concentrations were determined under conditions where a difference spectrum would show a maximum amount of reduced pigment. As noted above, this is realized by recording the difference between the anaerobic suspension and an aerobic sample with very low endogenous respiration. The respiratory inhibitor, Amytal, can also be used to oxidize pigments in the reference sample. This compound interrupts electron transfer between pyridine nucleotides and flavoproteins and thus leads to the reoxidation of pigments located on the oxygen side of its site of action, namely FP and cytochromes b, c, a, and a_3.

When Amytal is added to an aerobic suspension of starved bull spermatozoa, even at this low respiratory level a small amount of cytochromes b and c is reduced, approximating 10% of the total amount shown in Table I. With dog and *Spisula* spermatozoa, Amytal treatment of the reference sample proved to be an absolute requirement. In the case of dog sperm only a light washing was applied to avoid possible loss of pigments, particularly of cytochrome c. Respiration therefore remained high. The same situation prevailed in *Spisula* where the endogenous respiration of spermatozoa cannot be eliminated by repeated washing. With dog sperm 80% of the endogenous respiration is Amytal-sensitive, and a rather complete reoxidation of cytochromes may be expected, leading to reasonably good figures for cytochrome concentration. In *Spisula* sperm, however, as much as 50% of respiration (e.g., succinic acid oxidation) is resistant to Amytal. For the terminal cytochromes a_3, a, and c, which are usually less reduced in the aerobic steady state, the values in Table II may be considered correct. But values of cytochrome (b) are probably low, as are those for flavoproteins. Data for pyridine nucleotides are unreliable owing to the conditions of Amytal inhibition.

As seen in Tables I and II, there is no significant difference among the same bulls studied in different seasons or between dog and bull

TABLE 1. Relative proportions of respiratory enzymes, concentration, and turnover number of cytochrome a in bull spermatozoa

Series[a]	Components[b]							Av. Conc. of Cyt a, mμM/ 10^{11} Cells/l	Av. Turnover No. of Cyt a, with Substrates
	a_3	a	$c + c_1$	b	FP	PN	b_d	FP$_d$		
I	1.11 (0.82– 1.39)	1	0.61 (0.47– 0.83)	0.39 (0.22– 0.50)	2.37 (1.07– 3.00)	15.7 (15.7)	0.16 (0.10– 0.22)	0.70 (0.50– 0.96)	166 (150–195)	1.7 sec^{-1} (1.1–2.7)
II	1.12 (1.05– 1.29)	1	0.67 (0.54– 0.73)	0.36 (0.27– 0.47)	2.28 (1.58– 2.72)	14.3 (11.3– 20.3)	0.15 (0.15)	— —	144 (120–205)	1.3 sec^{-1} (0.7–2.1)

[a] Series I, average of 6 winter samples; II, average of 8 summer samples of sperm, all from bulls H 47 and H 43.
[b] Average values and range, in parentheses. Component with subscript d equals fraction not reduced by anaerobiosis but rather by dithionite.

TABLE II. Relative proportions of respiratory enzymes, concentration and turnover number of cytochrome a in dog and *Spisula* spermatozoa

Sperm	Components[a]						Av. Conc. of Cyt a, mμM/10^{11} Cells/l	Av. Turnover No. of Cyt a, with Substrates
	a_3	a	$c + c_1$	b	FP	PN		
Dog	0.94	1	0.67	0.32	3.4	—	120	4.8 sec^{-1}
Spisula	a_3 1.29	a 1	c 1.23	(b) 0.73	FP 1.26	PN 4.83	32	1.3 sec^{-1}

[a] Amytal present in reference cuvette.

spermatozoa. This applies to both the relative proportions and absolute concentrations of the cytochromes. The high proportion of cytochromes a and a_3, compared with other components of the respiratory chain, is unusual for mammalian cells and resembles somewhat the situation in muscle tissues of lower vertebrates such as the toad (Chance and Hess, 1959; Ramírez, 1959). The presence of cytochromes a and a_3 in equal amounts was further confirmed by spectra recorded after carbon monoxide treatment. To estimate the amount of cytochrome c_1, bull spermatozoa were exposed to distilled water for one hour at $5°C$, which resulted in a loss of cytochrome c without decreases in other pigments (Estabrook, 1958). Comparison of spectra obtained with an extracted sample and one treated only with saline showed that there is roughly a 1 to 1 ratio between cytochromes c and c_1.

In dog and bull sperm the cytochromes appear to exist approximately in the ratio, $3a_3 : 3a : 1c : 1c_1 : 1b$, and in *Spisula* sperm, $1a_3 : 1a : 1c : 1(b)$ (Gonse, 1959a,b,c). In the latter case, it is impossible to ascertain whether the (b) term represents one unit of $b(557)$ for one of $b(560)$; if such be the case, one of the b components could be considered as playing the part of cytochrome c_1 of bull or dog spermatozoa.

While the proportions between various cytochromes are different in these two kinds of spermatozoa, their respiratory activity is of the same order of magnitude as is shown by a comparison of concentration and turnover number for cytochrome a, designated TN a (Table II).

The TN a of *Spisula* spermatozoa is 1.3 sec^{-1}, which corresponds to a

Z_{O_2} of 0.9. In other preparations we observed Z_O values from 2 to 3, within the range of 1.6 to 4.0 as reported by Humphrey (1950) for oyster spermatozoa at the same dilution. The highest Z_{O_2} values for *Spisula* sperm would give a TN a of about 5 sec^{-1}, which is comparable to a TN $a = 4.8$ shown in Table II for dog spermatozoa (Z_{O_2} of 11.4). Washed bull spermatozoa, in the presence of lactate had a Z_{O_2} of about 3 in this study; values of 10 are reported elsewhere (Melrose and Terner, 1953) which would correspond to a TN a of about 6. All three spermatozoa thus have roughly identical turnover numbers for cytochrome a. When calculated for cytochrome c, the turnover number is about 15, well within the range of values found for other mammalian tissues (Chance and Williams, 1955). In *Spisula*, however, the TN c will be only 5 owing to the different proportion of cytochromes. By and large these figures are very close, and one can conclude that the respiratory chains in the three types of spermatozoa studied here operate at similar rates and that these rates are within the range of those of body tissues.

For the purpose of comparison, the values of molarity per cell (Table II) may not be very meaningful. Two other calculations have been made in an attempt to express results in terms of intra-mitochondrial molarity and number of molecules per cell (Table III). The necessary assumptions concerning sizes of mitochondria and middle piece are derived from figures and photographs given by Galtsoff and Philpott (1960) for oyster spermatozoa, Randall and Friedlaender (1950) for ram sperm, and Yasuzumi (1956) for rat sperm. Despite the fact that our figures for mitochondrial dimensions are the highest logically permitted, the values found for cytochrome

TABLE III. Cytochrome concentration and number of molecules in the spermatozoa of *Spisula* and bull

Sperm	Cytochrome a in M/10^{11} Cells/l	All Cyto-chromes in M/10^{11} Cells/l	Molarity of All Cyt in Mitochon-dria	No. of Mole-cules of All Cyt/Cell	Volume of Mitochon-drion	Assumptions
Spisula	32×10^{-9} M	136×10^{-9} M	3.02 mM	0.8×10^6	$0.45\ \mu^3$	4 spheres : 0.6 μ dia.
Bull	155×10^{-9} M	482×10^{-9} M	0.73 mM	2.9×10^6	$6.6\ \ \mu^3$	Sheath : 15 μ long, 0.9 μ ext. dia., 0.5 μ int. dia.

concentration are four to ten times larger than those reported elsewhere. In beef liver mitochondria, for example, Estabrook and Holowinsky (1961) obtained a figure of 0.1 to 0.5 mM for the concentration of all cytochromes along with flavoprotein. With an ultrasensitive spectrophotometer focused on a single cell Perry *et al.* (1959) found a total concentration of 0.14 mM in the giant mitochondrion or Nebenkern of the grasshopper spermatid. Two explanations can be offered for the apparent discrepancy with our results; either the cytochromes are located at sites other than the middle piece, or the mitochondrion of the mature spermatozoon is more condensed and has a higher internal concentration of enzymes than that of immature germ cells. Indeed, Nebenkern shrinkage is in fact very likely as the cell passes from spermatid to mature spermatozoa stages.

These results suggest that a more significant figure would be the number of cytochrome molecules per cell in relation to cell dimension, nitrogen content, or dry weight. Thus, the mature germ cells of the grasshopper and bull have about the same dimensions and, considering a Nebenkern of 30 μ^3, calculations from the data of Perry *et al.* (1959) give a value of 2.5×10^6 molecules of all cytochromes per cell, as compared with 2.9×10^6 found here for bull sperm. Humphrey (1950) gave a value of 1 mg dry weight per 10^8 oyster spermatozoa, the comparable value being 3 mg for bull sperm. By multiplying the figure 0.8×10^6 molecules per cell found in *Spisula* sperm by this factor, 3, one obtains 2.4×10^6 molecules per cell, again close to the figure for bull spermatozoa. It may be concluded, therefore, that the spermatozoa examined thus far carry equivalent quantities of cytochromes, namely about 2.5×10^6 molecules in each cell.

The above data indicate that while the relative proportion of cytochromes may differ significantly, no striking difference exists in either concentration or turnover number of the respiratory pigments of the two mammalian and one invertebrate species of sperm investigated here.

Function of Respiratory Enzymes

The response of the respiratory pigments to oxidizable substrates such as lactate or succinate has been studied on bull spermatozoa. A high positive effect of 20 mM lactate is observed on respiration of

mildly washed cells which usually do not respond to succinate; on more thoroughly washed spermatozoa, however, succinate evokes a tenfold increase in respiration, while lactate loses its effect. This can be interpreted as showing that extensive washing destroys both the normal impermeability to succinate and lactic acid dehydrogenase activity of the cell; succinic oxidase activity, however, persists. With the proper spermatozoan preparation and adequate substrate one always observes, on difference spectra at room temperature, the aerobic steady state which persists for a few minutes. A partial reduction of cytochromes a_3, a, c, b, of pyridine nucleotides, and of flavoproteins is detected after addition of lactate or succinate (Figs. 7 and 8). On low-temperature "stabilized steady states" cytochrome c_1 also appears involved.

The effect of antimycin A in the presence of lactate is also shown in Fig. 7. In this particular experiment respiration was inhibited by 87%. The increased reduction of cytochrome b or $b(d)$ is shown both here and in Fig. 6. Antimycin A is a specific inhibitor of electron transfer between cytochromes b and c_1 or c (Chance and Williams,
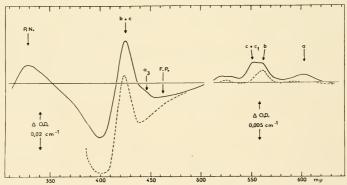
FIG. 7. Difference spectra of bull spermatozoa at 25°C. Cells washed and suspended in RP, pH 7.3. Sample H 513. Solid line: (lactate 20 mM, aerobic) − (aerobic, endogenous substrates). Dashed line: (antimycin A $4 \times 10^{-6}M$, lactate, aerobic) − (lactate, aerobic). TN a was 0.8 sec^{-1}. Reduction by lactate in per cent: 4.5 (cyt a_3), 13 (a), 35 ($c + c_1$), 41 (b), 23 (FP).

1956); it acts in low concentration, in some cases stoichiometrically in relation to cytochrome b (Chance, 1952). In experiments on dog and bull spermatozoa one finds a ratio of 2 to 3 antimycin molecules per cytochrome b equivalent (6×10^5 antimycin molecules per cell). This suggests that antimycin could be bound to components other than electron carries as is the case in ascites cells (Chance and Hess, 1959). This hypothesis is supported by the fact that antimycin A affects spermatozoan motility at concentrations lower than those required for inhibition of respiration.

The addition of Amytal, in the presence of succinate, does not lead to a significant change in the steady state of cytochrome reduction, and respiration is not inhibited (Fig. 8). Succinic acid oxidation nevertheless utilizes the normal respiratory chain from flavoproteins to cytochrome a_3, as shown when azide is added to a suc-
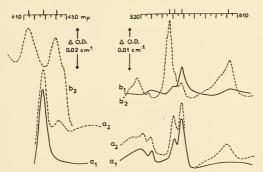
Fig. 8. Difference spectra of bull spermatozoa at liquid nitrogen temperature. Cells washed and suspended in RP, pH 7.2. Samples H4320 and H512. a_1 (solid line): (succinate 20 mM) − (endogenous substrates); both aerobic. a_2 (dashed): (succinate, Triton 3 o/oo) − (endogenous substances); both aerobic. b_1 (solid line): (succinate, Amytal 4 mM) − (Amytal 4 mM); both aerobic. b_2 (dashed): (succinate, Amytal, azide 10 mM) − (succinate, Amytal); both aerobic. Reduction by succinate in per cent: 3 (cyt a), 25 ($c + c_1$), 54 (b) in exp. a_1. Apparent lower reduction of cyt $c + c_1$ in exp. b_1 is due to Amytal effect on reference side. Reduction by azide in per cent: 54 (cyt a), 57 (c), 32 (c_1), 64 (b). Percentages obtained by comparison with sample of same preparation where pigments were totally reduced by anaerobiosis or dithionite.

cinate Amytal-treated sample. Respiration is then inhibited while terminal cytochromes are further reduced. Also shown in Fig. 8 is the peculiar effect of a detergent, Triton, which without increasing respiration brings about a significant increase in cytochrome oxidase reduction.

Azide appears to have a peculiar effect on the aerobic steady state of lactate oxidation in bull spermatozoa. From a combination of three spectra recorded on the same preparation at 25°C, one can calculate and obtain directly the percentage of reduction for pyridine nucleotides, flavoproteins, and cytochromes (Fig. 9). Azide has a multiple action; it increases the reduction of terminal cytochromes, a_3, a, and c, as one would expect from that inhibitor, but has no effect or rather a reoxidizing action on cytochrome b, while flavoproteins and pyridine nucleotides are more effectively reduced. The phenomenon occurring at the level of cytochrome b is better seen in low-temperature spectra recorded on "stabilized steady states" (Fig. 10). That cytochrome oxidase is a primary site of action for azide in bull spermatozoa is confirmed by the fact that the alpha absorption band of cytochrome a, normally at 600 mμ, is shifted to 596
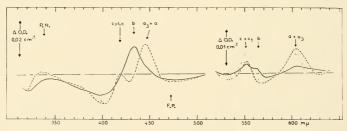
Fig. 9.   Difference spectra of bull spermatozoa at 25°C. Cells washed and suspended in RP, pH 7.2. Sample H4726. Solid line: (lactate 20 mM, aerobic) − (aerobic, endogenous substrates). Dotted line: (azide 10 mM, lactate, aerobic) − (aerobic, endogenous substrates). Dashed line: (azide, lactate, aerobic) − (lactate, aerobic). Reduction by azide in the aerobic steady state in per cent: 10 (cyt a_3), 30 (a), 25 ($c + c_1$), about 0 (b), 11 (FP), 19 (PN) calculated by difference between spectrum 2 and spectrum 1. Experimental check of those figures is directly afforded by spectrum 3 where reduction by azide is (%): 12 (cyt a_3), 28 (a), 25 ($c + c_1$), about 0 (b), 9 (FP), 14 (PN).

mμ in the presence of this inhibitor. At the same time, at liquid nitrogen temperature, the Soret band at 444 mμ is split into three components, an absorption maximum at 436 mμ and shoulders at 445 and 460 mμ. Assays on pure cytochrome oxidase by Yonetani and Bonner (1961) confirm this finding and demonstrate a splitting of the Soret band into two peaks at 436 and 445 mμ and troughs at 442 and 450 mμ. But azide action is apparently not confined to cytochrome oxidase, as is exemplified by the results on steady state of cytochrome b.

When azide is added to preparations treated with dinitrophenol and lactate, i.e., with high respiration, one again observes a further reduction of cytochromes a and c and a net reduction, rather than oxidation, of cytochrome b, contrary to the effect in the absence of DNP (Fig. 10, dotted curve). The effect of azide on the cytochromes
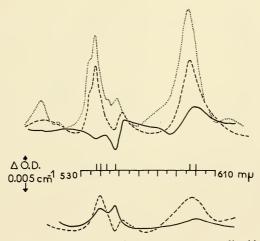
FIG. 10. Difference spectra of bull spermatozoa at liquid nitrogen temperature. Cells washed and suspended in RP, pH 7.2. Samples H4322, H4722. Lower curves: solid line: (lactate 10 mM, aerobic) − (aerobic, endogenous substrates); dashed line: (azide 10 mM, lactate, aerobic) − (lactate, aerobic). Upper curves: solid line: (DNP 0.1 mM, lactate, aerobic) − (lactate, aerobic); dashed line: (azide 10 mM, lactate, aerobic) − (lactate, aerobic); dotted line: (azide, DNP, lactate) − (DNP, lactate).
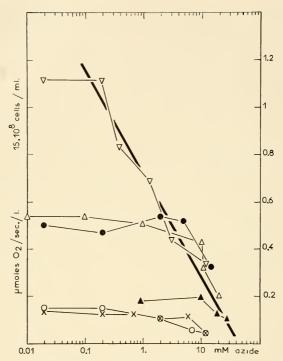
Fig. 11. Respiratory rates with increasing concentrations of azide shown for bull spermatozoa. Cells washed and suspended in RP, pH 7.2. Samples H4720, H4715, H4730. x, no addition, low endogenous respiration of washed cells; ○, addition of DNP 0.1 mM; ▲, addition of lactate 20 mM; ●, addition of succinate 20 mM; △, addition of lactate 20 mM and DNP 0.1 mM; ▽, addition of lactate 20 mM and DNP 0.2 mM.
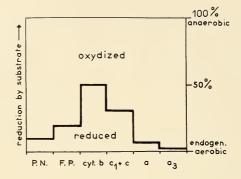
seems to be a function of the turnover of the preparation as seen more clearly with experiments on titration of respiration as a function of increasing azide concentrations (Fig. 11). These experiments were carried out on bull spermatozoa pretreated so as to be in the various metabolic states defined by Chance and Williams (1956). These are as follows: state 1, very low respiration of starved cells;

state 2, uncoupling by DNP without increase of respiration (no exogenous substrate); state 3, high respiration in the presence of exogenous substrate and uncoupling agent (DNP); state 4, intermediary respiration with substrate alone. In the preparations of spermatozoa the addition of azide gives inhibition which is dependent upon the rate of respiration or the turnover number as shown in Fig. 11 where results are computed for a given number of cells and therefore at constant cytochrome concentration. All titrations end along a line of azide sensitivity from 0.1 to 20 mM concentration. Samples with low respiration thus would appear not to be inhibited although spermatozoa do not carry any special azide-insensitive oxidative mechanism.

These observations are summarized in Fig. 12. The average level of reduction in the steady state of substrate oxidation (succinate or lactate) is shown in the upper section of the figure. The highest rate of reduction is observed with cytochrome b, which here plays, as in other types of cells, the role of bottleneck in electron transfer (Chance, 1955). However, it should be noted that pigments are not in equal amounts (i.e., cyt b is one-third of cyt a) and therefore, in absolute values, there is more flavine reduced, for instance, than cytochrome b in the steady state (see Table I).

Results from a series of experiments on azide action are grouped at the lower left of Fig. 12. The higher reduction of terminal components, cytochromes a_3, a, and c, is shown, as well as that of the initial ones, pyridine nucleotides and flavoproteins. In between, a phenomenon resembling uncoupling occurs at the level of cytochrome b. At this site there is a minimal reducing effect which at low turnover is in fact a reoxidation. This could be interpreted as due to the superimposition of two actions; first, the general increase of reduction following action of azide on cytochrome oxidase which is dependent upon turnover number, and second, a specific uncoupling exerted on cytochrome b. Other results are also in favor of an uncoupling action of azide, such as the data obtained on *Spisula* spermatozoa where respiration can be activated by azide.

Crossover points for antimycin A, Amytal, and DNP are given at the lower right of Fig. 12. Crossover points as defined by Chance and Williams (1956) are sites along the electron transfer chain where blocking occurs. Below such a point, on the substrate side, reducing
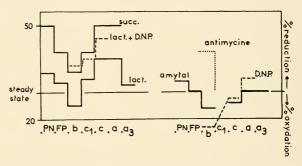
FIG. 12. Averaged results of spectroscopic records on bull sperma-
tozoa. Top: reduction of respiratory enzymes during steady state of lactic
or succinic acid oxidation; expressed in per cent of total amount of com-
ponent. Lower left: effect of azide on steady state in presence of succinate,
lactate, or lactate with DNP; expressed as percentage increase or decrease
from steady-state level of reduction. Lower right: effect of antimycin A,
Amytal, and DNP on steady state in presence of lactate; expressed as
percentage increase or decrease from steady-state level of reduction.

equivalents accumulate which lead to increasing reduction of pig-
ments. Above this point, on the oxygen side, oxidizing equivalents
are carried down and lead to oxidation of pigments. In bull sperma-
tozoa, with lactate as substrate, a crossover point for Amytal appears

between pyridine nucleotides and cytochrome b. With succinate as substrate, Amytal has no spectroscopic or respiratory effect. A cross-over point for antimycin A is located between cytochromes b and c_1, and for DNP between cytochromes c and a. The location of these points is, by and large, the same as that found in other cells or mitochondria.

Parallel with the spectroscopic studies, experiments were conducted on the effect of the same inhibitors on respiration and motility of *Spisula* and dog spermatozoa. Azide action on endogenous respiration of *Spisula* spermatozoa is, as in bull sperm, a complex one (Fig. 13). At concentrations from 1 to 3 mM, activation of respiration is noted, but some inhibition occurs at higher levels of azide. In some samples the phenomena balance one another, and there is no inhibition at 20 mM azide; in others, however, where activation had not occurred at 3 mM azide, inhibition is brought about by 20 mM azide, but at most this involves only a 50% inhibition of respiration. Motility decreases progressively for azide concentrations over 1 mM and is completely arrested by 20 mM azide. This inhibition of motility begins at concentrations which activate respiration, and thus points to an uncoupling effect of azide as was suggested by the data on bull spermatozoa. Here again, the high respiration obtained by the addition of DNP is more sensitive to azide than is endogenous respiration, and shows that inhibition is dependent on turnover number. As in the bull, at the end point of titration (20 mM azide), the noninhibited respiration, expressed in volume of oxygen consumed, is the same with or without DNP. The titration curve with azide in the presence of DNP follows a sigmoid pattern which could indicate that the secondary phenomenon, the activation of respiration, is superimposed here, too, but occurs at lower azide concentrations of 0.05 to 1 mM.

As in *Spisula*, inhibition of respiration by azide is not complete in dog spermatozoa; 30% of exogenous respiration is resistant to 10 mM azide (Fig. 14). Motility is progressively inhibited in the range 0.2 to 5 mM and completely lost at 5 mM azide. It is important to note that during the titration, the effect of azide on motility is not uniform. Some spermatozoa remain fully motile, others are completely arrested. As azide concentration increases, increasing numbers of immotile cells are found, a fact which indicates a threshold effect. This
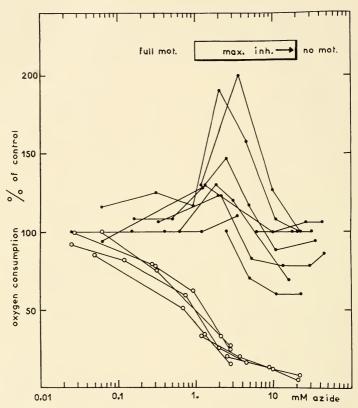
Fig. 13. Effect of azide on respiration in states 1 and 3 shown for *Spisula* spermatozoa. Cells in SW-GG, pH 7.3. Results from different preparations. ●, azide on endogenous respiration (Z_{O_2} of about 1); ○, azide in the presence of DNP, $0.5 \times 10^{-4}M$ ($Z_{O_2} = 5$ to 8). Within the frame, progressive inhibition of motility.

phenomenon is very different from that which occurs with other respiratory inhibitors. Also different is the fact that addition of glucose, absent from the seminal fluid of dogs, does not reverse the inhibition of motility. An action of azide on glycolysis therefore seems

likely, as suggested by certain investigations on other types of material (Chance, 1955; Clifton, 1946).

Thus, in *Spisula* as in dog spermatozoa, inhibition of motility by azide cannot be directly attributed to its inhibitory effect on respiration. Its apparent action as an uncoupler and as a glycolytic poison must be taken into account.

Amytal action on motility of dog spermatozoa parallels its inhibitory effect on respiration; 80 to 90% of respiration is Amytal-sensitive and motility is diminished considerably at 10 mM Amytal concentration (Fig. 15). In *Spisula* sperm where respiration is 50% Amytal-sensitive, motility is still normal at 10 mM Amytal and is affected only by higher doses (Fig. 16). In both types of spermatozoa, exogenous succinate failed to reverse partial inhibition of respiration, as it did in bull sperm, owing to a lack of permeability to this substrate. In any case, oxidation of endogenous succinate appears to
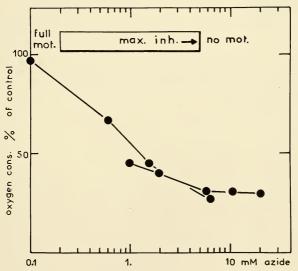
FIG. 14. Effect of azide on exogenous respiration of dog spermatozoa. Cells in seminal fluid with GG, pH 7.3. Z_{O_2} of 11. Within the frame, progressive inhibition of motility.
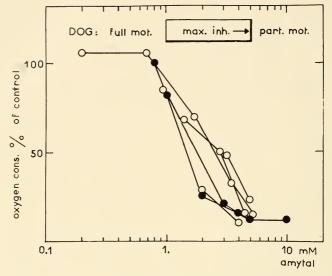
Fig. 15. Effect of Amytal on exogenous respiration. ●, dog sperma-
tozoa, seminal fluid with GG, pH 7.3; ○, bull spermatozoa, washed and
suspended in RP, pH 7.2, lactate 20 mM. Within the frame, progressive
inhibition of motility.

be responsible for a large part of the respiration of *Spisula* sperma-
tozoa.

During inhibition of motility by Amytal, all cells are affected at
the same time and to the same extent. With increasing Amytal con-
centrations, one finds a progressive decrease in frequency of tail beat,
the wave traveling more and more slowly along the flagellum, while
amplitude remains unmodified. Addition of glucose completely re-
verses Amytal inhibition of motility in dog spermatozoa. Another
observation recorded in Fig. 16 is the greater sensitivity to Amytal of
DNP-activated respiration in *Spisula* sperm. This indicates a possible
change in the nature of substrates utilized as induced by DNP.

With antimycin A, inhibition of motility of dog spermatozoa does
not parallel that of respiration (Fig. 17). Motility is affected at very
low concentration of the inhibitor, $2 \times 10^{-8} M$. As seen previously,
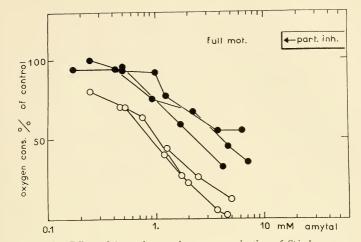
FIG. 16. Effect of Amytal on endogenous respiration of *Spisula* spermatozoa. Cells in SW-GG, pH 7.3. Results from 2 preparations. ●, Amytal on endogenous respiration (Z_{O_2} of about 1); ○, Amytal in the presence of DNP 0.05 mM (Z_{O_2} of 6 and 8). Within the frame, progressive inhibition of motility.
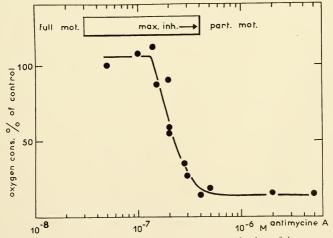
FIG. 17. Effect of antimycin A on exogenous respiration of dog spermatozoa. Cells in seminal fluid – RP (1:1), with GG, pH 7.3, 3.7×10^8 cells/ml, Z_{O_2} of 13. Within the frame, progressive inhibition of motility.

125

the molecular ratio between antimycin and cytochrome b, at 85% inhibition of respiration, is 2:1 in dog sperm, and therefore one could assume that the extra molecule of antimycin is first bound to a site which is more closely related to energy conservation, e.g., a transphosphorylating reaction, than to electron transfer. The addition of glucose, as in the case of Amytal, entirely reverses the inhibition of motility. No titration curves are available on *Spisula* spermatozoa. However, ethanol, which is currently used as a solvent for antimycin A, activates respiration of these spermatozoa when the concentration of alcohol exceeds 1%, a phenomenon not observed in dog sperm. Furthermore, in *Spisula* sperm, 2.5% ethanol produces a complete inhibition of motility. However, with $1.85 \times 10^{-6}M$ antimycin A (final ethanol concentration 0.75%) an inhibition of 50% of respiration is observed. This indicates an antimycin sensitivity of respiration in agreement with data of Bonner (1961) who found that the (b) cytochromes of scallops and *Spisula* muscles are both reduced by antimycin A.

From experiments with DNP, differences between spermatozoa from invertebrates and mammals are most clearly revealed. Sensitivity to the uncoupler varies widely (Fig. 18). In washed bull spermatozoa the average maximum activation of respiration by DNP is 2 times, with the optimum concentration of DNP 0.1 to 0.2 mM, when lactate has been added as substrate. With lightly washed dog sperm, the maximum activation was only 1.3 times normal respiration, which, however, is probably more significant than the higher effect found in bull. In *Spisula* spermatozoa the average of six experiments shows an activation of 6.8 times (up to 8.5 times in two samples). An experiment carried out on sperm of a different species of clam, Venus, yielded analogous results (Fig. 18). The optimum concentration of DNP for activation of respiration is 0.05 mM, lower than in either dog or bull sperm.

Motility in the spermatozoa studied is progressively inhibited at DNP concentrations which activate respiration, 0.02 to 0.12 mM in *Spisula* and 0.05 to 0.5 mM in dog sperm. Sensitivity varies widely, however; in *Spisula* sperm, motility can be completely arrested while in dog sperm a fair motility persists even in 1 mM DNP, so long as oxygen is present. Glucose completely reverses the partial inhibi-
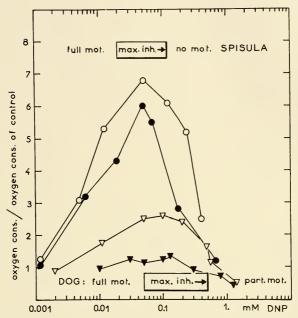
Fig. 18. Effect of dinitrophenol on respiration of different sperma-
tozoa. ▼, dog spermatozoa, seminal fluid with GG, pH 7.3; ▽, bull sper-
matozoa, washed and suspended in RP, pH 7.2, lactate 20 mM; ●, clam
spermatozoa (*Venus sp.*), in SW-GG, pH 7.3; ○, *Spisula* spermatozoa, in
SW-GG, pH 7.3. Within the frame, progressive inhibition of motility,
for *Spisula* (top), for dog (bottom).

tion of motility in dog spermatozoa, where "glycolytic motility" is
largely insensitive to oxidative metabolism and oxygen tension (Fig.
19). There seems to be no Pasteur effect on motility, a confirmation
of the data of Lardy *et al.* (1949) on lactic acid production by ejacu-
lated bull spermatozoa in aerobic or anaerobic conditions. However,
the reverse phenomenon, a control of oxidative motility by glycoly-
sis, is not unlikely in view of the fact that the presence of glucose
prevents the high response of motility to oxygen. This transient burst
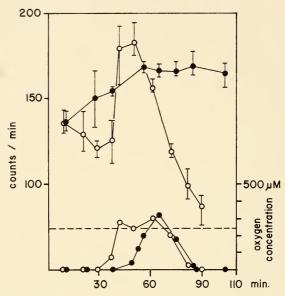
Fig. 19. Effect of oxygen on motility of dog spermatozoa. Cells sus-
pended in seminal fluid with GG, pH 7.3. Exp. J 83 and 84. Lower 2
curves: oxygen concentration (scale at right); the dashed line shows the
oxygen concentration in an air-saturated saline medium. Upper 2 curves:
relative ratings of motility on photoelectric counter; ○, seminal fluid, no
additions; ●, seminal fluid with 10 mM glucose added at time 0.

of motility which follows the sudden admission of oxygen into an
anaerobic medium deprived of sugar also shows the capacity of oxi-
dative metabolism to support full motility (Fig. 19).

CONCLUSION

Spermatozoa of the dog, bull, and clam, *Spisula*, possess full com-
plements of electron transferring enzymes in which the pigments are
of the same nature as those in the body tissues of the parent organism.
Concentrations of the components, when expressed in molarity in
the middle-piece mitochondria, are somewhat higher than in other

mitochondria as might be expected in such a compact cell. Calculation of the number of enzyme molecules per spermatozoon yields strikingly similar values for *Spisula* and bull gametes, despite important ecological differences between these spermatozoa. The activity of individual cytochromes, when defined by turnover numbers, are all within the same range and are comparable to values found with tissue mitochondria, ascites cells, and other biological preparations. A singular feature noted in spermatozoa of the dog and bull is the high relative content of cytochrome oxidase; this characteristic remains to be explained.

The active participation of these enzymes has been demonstrated during oxidation of lactic or succinic acid in bull spermatozoa. The respiratory chain offers no abnormal feature, responds to respiratory inhibitors such as Amytal and antimycin A with crossover phenomena which could be expected. Azide plays a complex role in spermatozoa, acting specifically on cytochrome oxidase, an effect currently associated with that inhibitor, but also possessing uncoupling action and possibly exerting an inhibition on glycolysis as well. The last two effects are primarily involved in the suppression of motility by azide. Such effects have either been observed or suggested in the course of other investigations bearing on different biological materials.

The important difference to be noted is in the response of spermatozoa from *Spisula* and dog or bull to an uncoupling agent, the spermatozoa of the invertebrate being much more sensitive. The effect of dinitrophenol on respiration is six times greater in *Spisula* than in dog sperm, and motility can be completely abolished in the former but not in the latter. Experiments with Amytal also show that in dog sperm, respiration and motility decrease in parallel, whereas in *Spisula* full motility is maintained at 60% inhibition of respiration. These results seem to indicate that in dog spermatozoa, conservation of energy associated with electron transfer is of less efficiency than that in *Spisula* gametes. Despite the capacity of oxidative enzymes to support motility and the higher theoretical yield associated with their activity, spermatozoa of mammals use glycolysis preferentially even in the presence of oxygen. An hypothesis to account for this is that of compartmentalization within the cell. Energy equivalents which appear through oxidative phosphorylation are

considered to be stored rather than used rapidly for motility; on the other hand, those derived from glycolysis are readily available for movement, and the carrier is recycled at a more favorable rate.

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Comments on Certain Aspects of the Metabolism of Spermatoza

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In our laboratory we have been interested in the metabolic processes of bovine spermatozoa, interrelationships in the metabolism of different substrates, and methods of regulating or controlling this metabolism.

Studies of some of the amino acids have been particularly interesting. Glycine has been shown to lengthen the time during which sperm remain motile when stored at 5° C (Roy and Bishop, 1954; Flipse and Almquist, 1956). Subsequent studies (Flipse and Almquist, 1958) have shown reduced accumulation of lactic acid but no reduction in the fructose which disappeared when the glycine buffer was used. Studies with glycine-C^{14} showed that glycine was converted to CO_2, although the glycine did not increase oxygen uptake above endogenous levels (Flipse and Benson, 1957). Labeled products from glycine, in addition to CO_2, included formate, glycolate, and glyoxylate.

These findings suggested the possibility of transamination, and we established this (Flipse and Anderson, 1959) by incubating sperm (and, in some instances, seminal plasma or dialyzed extracts of sperm) with labeled alanine and radioassaying the pyruvate recovered after incubation. In other experiments pyruvate-C^{14} was added as substrate and alanine recovered and assayed. Results of such studies are shown in Table I. First of all, interconversion of alanine and pyruvate was obtained, and secondly, the use of glycine buffer increased the conversion of pyruvate to alanine. Thus we postulated that the beneficial effect of glycine, in reducing lactate accumulation and in extending sperm livability, could be accounted for by the tendency

TABLE I. Effect of buffer on pyruvate-alanine conversion by washed bovine spermatozoa[a]

Product Assayed	Buffer (counts per minute)	
	Ringer Phosphate	Ringer Phosphate–5.0M Glycine (1:1)
Alanine	1100	1645
Pyruvate	1600	880

[a] Data from Flipse and Anderson (1959).

of the high glycine concentration to force the following reaction to the right:

$$\text{Lactate} \rightleftharpoons \text{Glycine} + \text{Pyruvate} \rightleftharpoons \text{Glyoxylate} + \text{Alanine}$$

Regardless of whether this reaction actually accounts for the observed extension of sperm livability, the results indicate that pyruvate is involved in sperm metabolism in more than the generally recognized glycolysis-respiration pathway.

This evidence of transamination in bovine semen prompted a search for other transaminase systems (Flipse, 1960a). Routine chemical methods were used for glutamic-oxaloacetic transaminase and glutamic-pyruvic transaminase, and both have been established as components of bovine semen. In fact, the glutamic-oxaloacetic transaminase level was found to exceed 600 units per milliliter of seminal plasma—some twenty times the concentration in normal blood serum. Additional assays revealed that the concentrations of both of these transaminases were at least five times as high in the spermatozoa as in seminal plasma.

Evidence has been presented by Dr. Terner in this symposium of the synthesis of diglycerides by spermatozoa. This is of considerable interest to us, for we have observed synthesis in our amino acid studies (Anderson and Flipse, 1959). Incubation of spermatozoa with histidine-C^{14} produced considerable activity in a compound which we eventually identified as the dipeptide carnosine. One is prompted to ask why synthesis occurs in these cells which are not subject to growth or division. Are not such reactions wasteful of the energy stores of the organism, and are they not likely to decrease survival time?

The relative importance of different substrates as energy sources is a subject with some interesting ramifications. Vantienhoven *et al.* (1952) reported that glucose was used in preference to fructose by bull spermatozoa, and we have confirmed this finding with C^{14}-labeled glucose and fructose. Our studies with octanoate-1-C^{14}, however, provide a different picture of substrate relationships (Flipse, 1960b). As shown in Table II, spermatozoa incubated with a constant amount of octanoate-1-C^{14} but an increased concentration of carrier octanoate produced $C^{14}O_2$ of reduced specific activity—the well-known isotope dilution effect. When unlabeled glucose replaced the unlabeled octanoate, a much smaller reduction in specific activity of CO_2 occurred, indicating that octanoate utilization is relatively independent of the presence of glucose. Perhaps more surprising was the observation that substitution of unlabeled acetate for unlabeled octanoate caused only a moderate reduction in specific activity of CO_2.

We have been reminded in this symposium of species-related differences in morphology of spermatozoa, and certain species-related differences in metabolism have been presented as well. It is important, of course, that we specify the species when discussing the importance of a particular metabolic reaction. It is also important to note the condition of the preparation being used; Dr. Salisbury has presented data clearly illustrating this point. I am often reminded of an experience in our laboratory a few years ago when we were studying spermatozoan metabolism of glucose-C^{14}. We were using washed sperm in these studies, and it so happened that on one oc-

TABLE II. Production of $C^{14}O_2$ from octanoate-1-C^{14} by washed bovine spermatozoa during 1 hour of incubation at 37°C in modified Ringer's solution pH 7.2[a]

Concentration ($\times 10^{-3}$ M)	Unlabeled Substrate (counts per minute per micromole of $C^{14}O_2$)		
	Octanoate	Glucose	Acetate
0	650	650	650
1	220	550	480
5	45	560	410

[a] Data from Flipse (1960b).

casion the sperm were found immotile after the preparative wash-
ing. Rather than discard the preparation, we followed through the
outlined experimental procedure to determine whether immotile
sperm show metabolic activity. To our surprise the $C^{14}O_2$ produc-
tion by immotile sperm was almost twice that normally observed
with motile sperm, whereas the yield of labeled lactate was one-
fourth the normal amount. This, I think, illustrates the importance
of specifying the age, motility, method of preparation, and other ap-
propriate conditions which might apply in studies of the metabolism
of spermatozoa.

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A Theory of the Survival Value of Motility

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The widespread occurrence of motile forms strongly suggests that the ability to move has, in some species at least, increased their chance of survival. Certainly it can be argued that, in general, motile forms can escape from predators more readily, collect food more rapidly, and find a mate more frequently than their nonmotile counterparts. As a consequence of these improved capabilities, their chances of survival are greater. These are reasonable and plausible explanations of the survival value of motility, but they lack decisiveness. I shall present here a heuristic approach to the development of the relationships which describe the energetics of motility of organisms which move through liquids at Reynolds numbers considerably less than one. The relationships will then be used as a basis for understanding the survival value of motility. Since, for the most part organisms whose movements are characterized by low Reynolds numbers are Protozoa or microorganisms, they will be referred to as such in what follows.

To begin with, we shall eliminate from further consideration all hypotheses of the basis of the survival value of motility except the one that motility increases the rate of food collection and, hence, the rate of energy accumulation by the organism. This does not imply that the other hypotheses are not applicable in some cases, for no doubt they are. It is clear, however, that they lack generality. Many microorganisms do not mate, and motility can hardly be expected to increase the chances of mating if it never occurs. Escape from a predator or an unfavorable environment presupposes that the individual is aware of the fact that he is being chased or that he is in an unfavorable environment. It is unlikely that all Protozoa and microorganisms can

detect the presence of predators or sense a deleterious environment, although it is certainly true that some can. Even so, the speeds with which they travel, tens to hundreds of microns per second, are hardly enough to get them away from some of the faster swimming micro-organisms. In fact, in the case of bacteria which are subject to preda-tion by bacteriophage, a predator that probably does not sense its prey until it collides with it, it is clear that in the presence of a horde of phage particles the execution of a random, helter-skelter escape pattern on the part of the bacterium will only increase the chances of its being caught because such a motion increases the probability of collision with a phage particle. In the presence of phage the bac-terium is less likely to get caught if it remains completely motionless. Still another reason for not considering these arguments is the fact that they do not readily lend themselves to a formal analysis of the type that can be expected to lead to conclusive results. True, it is possible to make appropriate simplifying assumptions but in so do-ing one is very likely to weaken the analysis to such an extent as to render it of little real significance.

On the other hand, the need to collect nutrient substances from the surroundings in order to survive is common to all forms. The viruses constitute a possible exception but even in this instance growth, if not survival, requires the presence of living cells. On the basis of generality, therefore, the argument that motility increases the ca-pacity for the organism to collect food is the only one that is not readily ruled out. While it is obvious that motility increases the capacity to collect food, it is not obvious that at the same time it always, or even ever, results in a net increase in the energy stores of the organism; for there can be no doubt that the process of moving itself requires energy, and unless there is a net gain in energy above the minimum required for existence, there can be no net growth, and indeed the organism may eventually commit suicide by continuing to move. From the fact that motile forms do exist we can conclude of course that there are conditions under which it is possible to collect more energy by moving than the process of moving requires. This does not, however, tell us what the conditions are. We seek here to develop these conditions.

A MODEL SYSTEM

To understand the energetics of motility it is convenient to con-
sider a model system which possesses the essential characteristics of a
single microorganism and at the same time lends itself to a formal
analysis. We take as our model a single spherical metabolizing cell,
in a liquid bath containing all the substances essential for life and
growth. The cell is assumed not to require mating for growth, and
the environment is taken as free of predators and noxious agents. We
then ask, how does the rate of food (or energy) collection and the
rate of energy expenditure depend on the average velocity with which
such a cell moves through the liquid when the motion is such that
the Reynolds number of the system is much less than one?

THE STATIONARY METABOLIZING SPHERICAL CELL
IN AN UNSTIRRED BATH

Consideration will be given first to the case of the stationary me-
tabolizing spherical cell in an unstirred bath in order to gain some
understanding of the factors that determine the rate of food collec-
tion in the simplest possible case. The only process by which nutrient
substances can get to such a cell is by diffusion, of course. The con-
centration of those substances metabolized by the cell is reduced at
the surface of the cell, and a concentration gradient is established.
The gradient develops both in space and time until a steady state of
diffusion is reached in which nutrient molecules diffuse to the cell
surface at a rate just equal to that at which they are metabolized by
the cell. Since the lowest value that the concentration of any sub-
stance can have at the surface of the cell is zero, the maximum
steady state rate of diffusion to the cell surface will be obtained for
this condition. Our problem then is to develop from diffusion theory
the relation for the steady state rate of flux of diffusing substance
across a spherical surface of given radius. This is the problem of the
spherical sink that is treated in almost every text on diffusion processes.
To solve it one considers a stationary sphere of radius R at the origin.
At time zero, the concentration of the diffusing substance is taken as
zero for all values of the radial coordinate r, less than or equal to R,

and it is taken as C_0 for all values of r greater than R. The solution to the diffusion equation for this case is

$$C = C_0 \left[1 - \frac{R}{r} + \frac{2R}{r\sqrt{\pi}} \int^{(r-R)/2(D_n t)^{1/2}} \exp\left(-x^2\right) dx \right]$$

where C is the concentration, D_n the diffusion coefficient of the nutrient, and t the time.

The flux Φ across the surface $r = R$ is

$$\Phi = 4\pi D_n \left(r^2 \frac{\partial c}{\partial r} \right)_{r=R} = 4\pi D_n R C_0 \left(1 + \frac{R}{(\pi D_n t)^{1/2}} \right)$$

as $t \to \infty$, $\Phi \to \Phi_s = 4\pi D_n \cdot R \cdot C_0$, the steady-state flux of nutrient across the cell boundary.

The significance of this result should be examined before proceeding further. The rate at which the stationary cell collects a particular nutrient from an unstirred medium depends directly on the concentration of the nutrient, C_0, the diffusion coefficient of the nutrient, D_n, and the radius, R, of the cell itself. Clearly the cell can do nothing to control the value of C_0, the concentration of nutrient in the environment. It is by virtue of the dependence of Φ_s on C_0 that the cell is at the mercy of its environment. If C_0 falls so low that Φ_s drops below the minimum value for survival the cell dies or, if possible, becomes dormant. Faced with a decrease in C_0, the cell might increase Φ_s by increasing its radius R. All other things remaining the same, a cell can increase its capacity to collect food in direct proportion to an increase in its radius. It is doubtful that a cell can increase in size without at the same time increasing its basal metabolic rate. An increase in size would not be of much help if the demand for nutrient increases as rapidly as, or more rapidly than, the increase in the supply. Nothing would be gained by getting bigger in such a situation. There is, however, a way in which the radius can in effect be increased and that is by local stirring. Obviously the organism cannot stir the entire bath, but conceivably it could be equipped with some kind of organ, flagella perhaps, which with a small expenditure of energy could stir the fluid in the immediate vicinity of the cell. Such stirring would convey all nutrient molecules that entered the stirred region to the surface of the cell where they would be metabolized. The effect of local stirring would be to increase the

effective value of R at which the concentration of nutrient is reduced to zero, and hence increase there maximum flux available.

I submit, therefore, that there may be cells which possess flagella, or other similar structures, which beat asynchronously and do nothing more than stir the surrounding fluid and thereby increase the steady-state flux rate of nutrients to the cell surface. Indeed, this may even be the major function of flagella. It is my guess that such a stirring mechanism might be expected to produce an increase of as much as an order of magnitude or so in the steady-state flux rate, for if the stirring structures have roughly the same dimensions as the cell and their motion is characterized by a low Reynolds number they will, in the first approximation, stir effectively only that solution which is not too far removed from the region through which they sweep.

THE CASE OF THE MOVING CELL

It has been seen that, faced with a steadily decreasing supply of food, that is, a drop in C_0, a given steady-state influx of nutrient can be maintained by increasing the effective radius of the cell, all other things being held constant. What else can be done? The only other term in the expression for the flux of nutrient across the cell surface which is available for manipulation is the diffusion coefficient of the nutrient. At first sight it would seem that there is nothing much that can be done about this except for the cell to evolve in such a way that it requires only those molecules which have the highest values for their diffusion coefficients, namely the smallest molecular species. It turns out, however, that there is something that can be done to increase the effective D_n and that is to move.

First let us consider the simple case of an organism which moves with a constant linear velocity, v. In addition to those molecules which reach it by diffusion, it will collide with and capture those molecules which lie within the volume that it sweeps out in a unit of time. The volume swept out per unit time is $\pi \cdot R^2 \cdot v$. By assuming 100% capture efficiency the number of molecules intercepted per unit of time Φ_v is given by the volume swept out times the concentration, that is,

$$\Phi_v = \pi \cdot R^2 \cdot v \cdot C_0$$

By assuming in the first approximation that this increased flux adds directly to the flux due to diffusion, the steady-state flux for the moving cell becomes

$$\Phi_s = 4 \cdot \pi \cdot RC_0 \cdot \left(D_c + \frac{R \cdot v}{4} \right)$$

If the cell executes not a linear but a random motion which can be characterized by a "diffusion coefficient," D_c, then, as was shown by Chandrasekhar (1943) the flux relation becomes

$$\Phi = 4\pi \cdot RC_0 (D_c + D_n) \left(1 + \frac{R}{\left[\pi(D_n + D_c)t \right]^{1/2}} \right)$$

where D_c is the diffusion coefficient of the cell's motion. For $t \to \infty$, Φ becomes

$$\Phi_s = 4 \cdot \pi \cdot rC_0 (D_c + D_n)$$

The diffusion coefficient of the cell is defined in terms of its motion as

$$D_c = \frac{\langle r^2 \rangle}{6\tau}$$

where $\langle r^2 \rangle$ is the mean square displacement in the time τ. $\langle r^2 \rangle$ is determined by the statistical properties of the motion of the cell. D_c can be related to what might be considered an average velocity of the particle by noting that

$$\frac{\langle r^2 \rangle}{6\tau} = \frac{\langle r^2 \rangle^{1/2}}{\tau} \cdot \frac{\langle r^2 \rangle^{1/2}}{6} = v \cdot \frac{\langle r^2 \rangle^{1/2}}{6}$$

where $v = \langle r^2 \rangle^{1/2} / \tau$ is the average velocity of the cell. This leads to

$$\Phi_s = 4\pi \cdot RC_0 \left(D_n + v \cdot \frac{\langle r^2 \rangle^{1/2}}{6} \right)$$

for the flux of nutrient.

In summarizing the situation for the moving cell it can be stated that the flux of nutrient across the boundary will in the first approximation depend linearly upon the velocity v, that is, $\Phi_s = 4\pi \cdot RC_0 \cdot$

$(D_n + K \cdot v)$, where the constant K will depend on the kind of motion executed by the particle.

THE DEPENDENCE OF ENERGY EXPENDITURE ON THE AVERAGE VELOCITY OF THE CELL

Now comes the question of the dependence of the energy expended by the cell velocity. A consideration of the hydrodynamics of the motion of microorganisms reveals, as was pointed out by Taylor (1951), that these motions are characterized by low Reynolds numbers. The Reynolds number provides an estimate of the relation of the inertial stresses in the fluid surrounding the moving organism to the viscous stresses and is given by, $l \cdot v \cdot \rho / \mu$, where l is length of the particle, v is its velocity, ρ is the density of the fluid, and μ is the viscosity of the fluid. For most microorganisms this quantity is less than 10^{-2} or 10^{-3}. This means that the inertial stresses are less than 1% of the viscous stresses. For such a situation one can neglect the inertial forces in the hydrodynamic equations of motion and consider only the viscous forces. It has been shown quite generally by Lamb (1932) that motions dominated by viscous forces dissipate energy in proportion to the square of the strain rate and hence to the square of the velocity, that is, the rate of energy dissipation, P_m, due to the motion at low Reynolds number, is given by a relation of the form, $P_m = A \cdot v^2$, where A is a constant that will depend on the particular motile process involved.

To determine the net rate of energy dissipation by the cell, allowance must be made for the fact that the motility mechanism is not 100% efficient. For every unit of energy converted to motile energy, and thence dissipated as heat in the surroundings, there is a certain amount of energy lost directly as heat owing to the inefficiency of the motility machine. In addition to this energy dissipated as a result of moving, there is also the basal rate of energy expenditure which is essential for survival of the cell. If we let P_0 equal the basal rate of energy expended by the cell, and let E designate the efficiency of the motility mechanism, then the total energy dissipated by the moving cell becomes

$$P = P_0 + \frac{A}{E} \cdot v^2$$

THE BALANCE OF ENERGY EXPENDITURE
AND ENERGY ACCUMULATION

Having developed relationships for the dependence of the rate of nutrient accumulation and the rate of energy expenditure on velocity with which the cell moves, we are in a position to examine the conditions of energy balance for the moving cell.

This is most easily done with the aid of some graphical plots of the relations

$$\Phi_s = 4\pi \cdot RC_0 (D_n + K \cdot v)$$

and

$$P = P_0 + Av^2/E$$

A plot of Φ_s as a function of v is a straight line with a slope, $4\pi \cdot RC_0 \cdot K$; a y-axis intercept, $y_0 = 4\pi \cdot RC_0 \cdot D_n$; and an x-axis intercept, $v_0 = -D_n/K$. A plot of P as a function of v is a parabola with a y-axis intercept of P_0. In Fig. 1a, Φ_s and P are plotted against v. In this plot C_0 is such that the intercept $y_0 = 4\pi RC_0 D_n$ is greater than P_0. This simply means that the concentration is such that the maximum rate at which nutrient can diffuse to the cell is greater than the basal metabolic rate P_0. The cell is not limited in its supply of nutrient by diffusion; hence growth is possible. Movement is not necessary in order to increase the influx of nutrient. The cell can, however, move with velocities v_m or less and remain in energy balance, although there is a velocity somewhat less than v_m which if exceeded will result in a decrease in the influx of nutrient over that which can be attained at rest.

In Fig. 1b the concentration has decreased so that now $y_0 = P_0$. The stationary cell receives, by diffusion, barely enough nutrient to survive. There is no nutrient left over for growth. Under these circumstances the cell can increase its influx of nutrient by moving at velocities less than v_m.

In Fig. 1c the concentration has dropped still farther and now y_0 is less than P_0. The flux of nutrient by diffusion to the stationary cell is insufficient for survival. The cell must move with a velocity v_b if it is to survive and with a velocity greater than v_b but less than v_m if it is to grow.

In Fig. 1d the concentration of nutrient has dropped to the point

where moving with a velocity v_b just enables the organism to survive. Energy is no longer available for growth, and the species is doomed to extinction unless a more efficient method of collecting nutrient can be developed.

On the question of the efficiency of the motility mechanism it is important to note that, *no matter how inefficient the motility mechanism is, it is always possible by moving to achieve a net gain in the collection of nutrient.* This is a direct result of the parabolic relationship for energy expenditure and the linear relationship for energy accumulation. The zero initial slope of the parabolic relation guarantees that *no matter what the efficiency E of the mechanism, there will always be a range of velocities for which the energy intake will exceed the energy expended.* Of course as the concentration of nutrient falls in the environment, there will
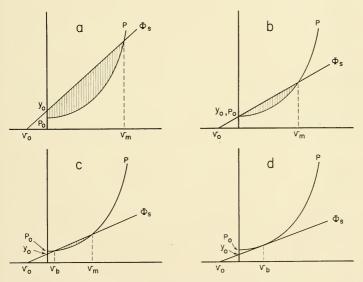
Fig. 1. Energy accumulation and expenditure diagrams for a motile organism. In going from plot *a* to *d* the concentration of substrate in the environment decreases progressively and gives rise to the indicated changes in the intersection of the $\Phi_s - v$ line with the $P - v$ curve. For explanation see text.

be selection in favor of those forms which have the most efficient motile mechanisms.

This analysis can hardly be considered as complete, for it has oversimplified the problem of diffusion to a moving cell, and stirring in the environment, such as undoubtedly exists in nature, has been neglected entirely. There are, however, a few new results that should be emphasized. The first is the suggestion that flagella may function primarily as agents for stirring of the local environment and thus increase the rate of influx of a required nutrient. Motility would in this case be incidental. The second significant result is that, no matter how inefficient the mechanism of motility may be, it is always possible to achieve a net gain in energy intake by moving. And finally, in an environment with limited or decreasing supply of essential nutrient, all other factors being equal, those organisms with the most efficient motile mechanisms will tend to be selected for by virtue of their ability to collect extra nutrient for growth at a higher rate.

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Sperm Tail Structure in Relation to the Mechanism of Movement[*]

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Efforts to relate sperm tail structure to the mechanism of sperm locomotion are complicated by the lack of unanimity as to whether the tail movements are planar undulations or three-dimensional gyrations. The intermittent flashing of the flattened heads of free swimming bull sperm, when observed in dark-ground illumination, was interpreted to mean that the heads rotated as the sperm swam (Rothschild, 1953). This fostered the belief that the tails execute spiral movements. More recent dark-ground cinephotographic studies of the simple flagellate sperm of invertebrates by Gray (1955) have shown that with slow shutter speeds a luminous region is recorded behind the head which delimits the area within which the tail movements occur. As the sperm head oscillated about its long axis in swimming, this "optical envelope" of the tail alternated between an elliptical and a linear configuration. Since the optical envelope of a tail executing helical movements would be expected to approximate a cylinder, it was thought that the observed alternation of its shape during swimming could best be explained on the basis of a two-dimensional wave motion. Similar studies on bull sperm, which have a more complex tail structure, yielded results which suggested that in this species, too, the movements were predominantly two dimensional, but there appeared to be a slight torsional component such that points near the tip of the tail followed a flat figure-of-eight path (Gray, 1958). Other analyses of cinematographic and photoelectric measurements on bull sperm (Rikmenspoel, 1958) have led to very different conclusions, namely, that the sperm cell does rotate

and the tail wave is three dimensional. The nature of the sperm tail movements thus continues to be a subject of dispute.

The early electron microscopic studies of motile cell processes in cross section revealed that the axial filament complex of cilia and of sperm flagella is essentially identical, consisting of two central, single filaments surrounded by nine evenly spaced, double filaments (Bradfield, 1953; Fawcett, 1954). This finding posed the difficult problem of accounting for the planar motion of cilia and the helical motion of sperm tails with the same internal arrangement of filaments. Ingenious explanations were offered which assumed, in the case of the cilium, a contraction of filaments on alternate sides to produce pendular oscillations, and in the case of the sperm tail, a sequential activation of the nine outer filaments initiating waves of shortening that are propagated along the filaments out of phase so as to produce helical movements (Bradfield, 1955). For this explanation to hold, it is necessary to assume the existence of two quite different mechanisms at the base responsible for the two distinct patterns of coordination in the contraction of the several core filaments. No morphological basis for such a difference has been found. The problem of seeking a single satisfactory explanation for both ciliary and sperm tail movements is greatly simplified if one adopts the view that sperm tails, like cilia, execute bending movements that are mainly in one plane. The fact that in sperm tails the bending movements result in waves that are propagated from base to tip can, in part, be regarded as a consequence of their considerably greater length. In the discussion which follows, we shall proceed from the assumption that sperm tail movements are mainly two dimensional, although we realize there are some who will not share this view.

THE AXIAL FILAMENT COMPLEX

Since the description of the basic nine-plus-two fiber pattern of cilia and flagella about eight years ago (Manton, 1952; Fawcett and Porter, 1954), several additional details of the fine structure of the fibrils and of the interfibrillar matrix have been discovered. Afzelius (1959), working with sea urchin sperm tails, drew attention to a slight inequality in the size of the two members of each doublet and noted that the line through their centers was not on the circumfer-

ence of a circle but inclined inward a few degrees. He also described small "arms" projecting from one member of each doublet toward the next in the row and observed a spokelike pattern of linear densities radiating from the central pair toward the nine fibers of the outer row. These findings have been extended by Gibbons and Grimstone (1960) in an elegant study of protozoan flagella. The presence of arms was confirmed, and it was established that they are two in number, and are consistently oriented in a clockwise direction from the point of view of an observer looking along the flagellum from the base toward the tip. The subfiber bearing the arms was designated subfiber A and the other, subfiber B. Instead of a spokelike radial pattern in the matrix, these authors observed in heavily stained cross sections of flagella, nine dots situated midway between the central and the outer fibrils (Fig. 1A). These were interpreted as end-on views of an additional set of slender longitudinal filaments overlooked by
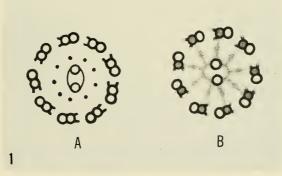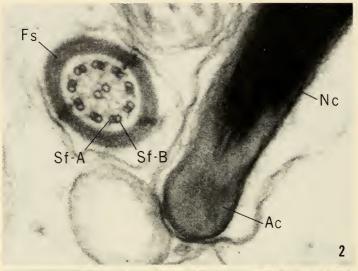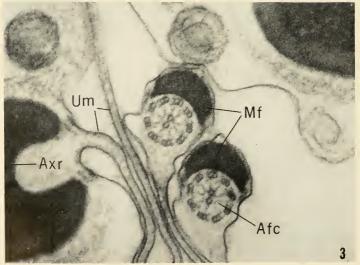
Fig. 1.   A. The structure of the *axial filament complex* of a flagellum as depicted by Gibbons and Grimstone. The central pair of fibers is enclosed in a central sheath and nine *secondary fibers* are situated midway between the central pair and the outer double fibers. B. The author's interpretation of the axial filament complex of sperm flagella. Instead of a second set of fibers there is a pattern of linear densities in the matrix consisting of two curved lines connecting one member of the central pair to the other, and nine radial lines connecting the central pair with subfiber A of each outer fiber. Subfiber A of each doublet bears a pair of short arms and its interior is considerably denser than that of subfiber B.

previous investigators. A delicate line around the center fibrils was believed to be a spirally wrapped filament or membranous sheath enclosing the central pair of fibrils.

Our own recent observations of amphibian and mammalian sperm tails confirm the presence of pairs of "arms" on subfiber A and reveal a significant difference in the density of the two subfibers of each doublet (Fig. 1B). A slightly greater density in the interior of subfiber A was noted in the protozoan flagella studied by Gibbons and Grimstone (1960) and a more noticeable difference has recently been reported by Nagano (1960) in rooster sperm tails. The density of subfiber A is quite marked in the sperm tails of rat, guinea pig (Figs. 5–7), and bat (Figs. 2 and 8). Indeed this subfiber may appear homogeneous in cross section whereas subfiber B appears tubular because it has a dense limiting layer around a center of low density. In the salamander sperm, on the other hand (Fig. 3), the subfibers both appear tubular, and a difference in density of their interior is scarcely detectable.

To date we have been unable to confirm the existence of a central sheath or of a set of secondary filaments. Instead we find the central pair of fibrils connected on either side by arcuate densities in the matrix which do not seem to constitute an enveloping sheath (Fig. 1B). Other linear densities extend radially from these two curving lines or from the central fibers themselves to subfiber A of the nine

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Fig. 2.   An electron micrograph of a small area of the lumen of the convoluted epididymal duct of a bat, *Myotis lucifugus*, showing, at the right, a portion of the head of one sperm with the tip of its nucleus (Nc) and acrosome (Ac); and, at the left, the tail of another spermatozoon cut transversely through the principal piece. The axial filament complex is surrounded by a thick *fibrous sheath* (Fs). Subfiber A (Sf-A) of each doublet is distinctly denser than subfiber B (Sf-B). A faint radial pattern of linear densities is discernible in the interfibrillar matrix.

Fig. 3.   Transverse sections of sperm tails of *Triturus viridescens*. The undulating membrane (Um) arises as a broad, thin fold of the plasma membrane attached at its base to the margins of a deep groove in the axial rod (Axr) which is the core structure of the tail. The axial filament complex (Afc) and marginal fiber (Mf) are found in the free edge of the undulating membrane. The spokelike arrangement of linear densities connecting the central pair to the peripheral doublets is especially clear in this material.

peripheral doublets. The mid-portion of each of these radial laminae is slightly thickened. Our observations are based mainly upon the examination of unstained cross sections (Fig. 2 and 3). Although the micrographs lack the high contrast and diagrammatic clarity of the elegant micrographs of protozoan flagella published by Gibbons and Grimstone, it is quite possible that they retain more detail. When heavy metal staining is used and every photographic device is employed to gain contrast, the more delicate portions of the radial pattern of connections in the matrix may be lost and only the thicker central portions of the dense laminae are then recorded as a series of dots midway between the central and peripheral fibrils. This may be the basis of Gibbons and Grimstone's conclusion that there is a set of secondary filaments interposed between the central and peripheral fibrils.
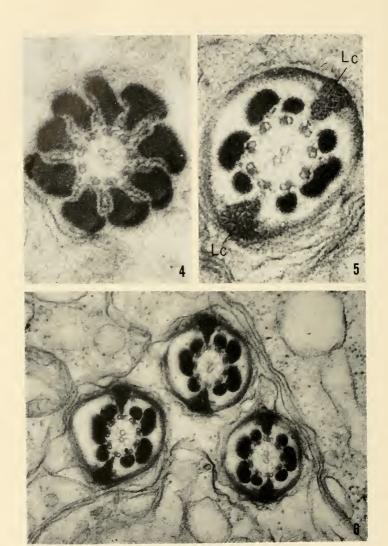
THE OUTER COARSE FIBERS

A characteristic feature of mammalian sperm tails is the presence of an extra outer row of nine coarse longitudinal fibers that are lacking in the sperm tails of fish and many of the invertebrates. The internal structure of the tails of invertebrates is essentially identical to that of flagella and cilia generally. Instead of the familiar pattern of nine-plus-two fibrils, the mammalian sperm tail has nine, plus nine, plus two, or a total of 20. The fibers of the outer row are very dense, homogeneous, and evidently composed of a substance quite different from that of the inner fibrils. Except for a few observations on their solubility properties and resistance to enzymatic digestion (Bradfield, 1955), very little is known about the physical properties or chemical nature of these fibers. It is generally assumed that they are accessory contractile elements possibly serving to overcome the resistance to bending offered by the relatively thick mitochondrial and fibrous sheaths, thereby strengthening the propulsive force of the tail. The nine coarse fibers vary in their thickness and cross-sectional shape. The differences are least conspicuous near the junction of the head and tail (Fig. 4) but become more obvious in the midpiece and principal piece. In several common rodent species three of the fibers are distinctly thicker than the others. It will facilitate description to be able to designate these fibers by number.

In so doing, we adhere to the system of numbering adopted for cilia and flagella by Bradfield (1955), and Afzelius (1961), as follows. A plane perpendicular to the line joining the midpoints of the two central fibrils divides the axial filament complex into equal halves with four fibrils on each side and one unpaired fibril lying in the plane of bilateral symmetry. It is customary to call the unpaired fibril *one* and to number the others in clockwise sequence. In the mammalian sperm there is a coarse outer fiber for each doublet in the axial filament complex, and these are given corresponding numbers. It is numbers 1, 5, and 6 of the outer row that are usually the largest (Figs. 5–7). If the plane of bending of the sperm tail is perpendicular to the line (XY in Fig. 7) through the central pair of fibrils, as appears to be the case for cilia (Fawcett and Porter, 1954) then the three largest fibers of the outer row are in the most favorable position to contribute effectively to movement in that plane.

The nine outer fibers are thickest in the first part of the midpiece but become thinner farther along the tail and terminate at different levels. Their length varies considerably from one species to another. In man and monkey they rapidly diminish in thickness in the midpiece and end in the proximal part of the principal piece. In rat, mouse, and guinea pig, on the other hand, they taper more gradually and extend throughout the greater part of the principal piece. A systematic study of transverse sections of the sperm tails of these species (Fawcett, 1961; Telkka *et al.* 1961) reveals that the nine fibers are of different lengths and that they terminate in pairs in a regular sequence along the length of the tail. Fibers 3 and 8, which are relatively small in diameter at the outset and are least favorably located to contribute to the bending movements, end first in the upper part of the principal piece. The remaining fibers terminate at points farther along this segment in order of increasing size. Four and 7 drop out, then 2 and 9, while 1, 5, and 6, which are largest at the outset, continue nearest to the distal end of the principal piece. The observation that those three fibers, which are best situated to contribute to bending in the presumed plane of undulation of the tail, are both the thickest and the longest favors the belief that the dense peripheral fibers of the tail are contractile elements.

Further support for this supposition has come from studies of Cleland and Rothschild (1959) on the bandicoot spermatozoon. The

midpiece and principal piece of the tail are unusually thick in this species. Electron micrographs of transverse sections show that the nine coarse fibers do not vary appreciably in thickness and unlike those of more flexible sperm, they are located a considerable distance from the axial filament complex. Fibers 1, 5, and 6 in our system of numbering are farthest from the axis of the tail, and 3 and 8 are nearest. This outward displacement of the peripheral fibers was interpreted as an adaptation to increase their effectiveness as tensile elements by placing them farther from the hinge point (Cleland and Rothschild, 1959).
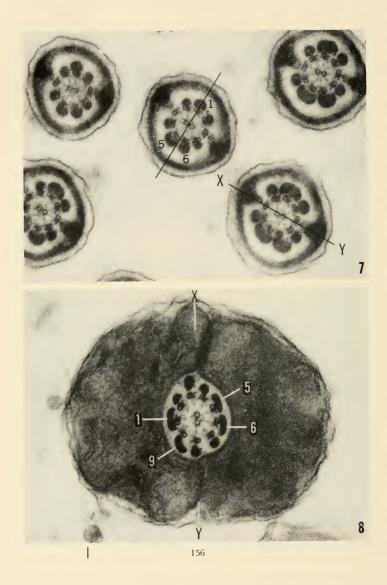
THE MITOCHONDRIAL SHEATH

Among the points that remained controversial at the level of resolution of the light microscope was the question as to whether the

Fig. 4. Transverse section of a late spermatid of guinea pig in the beginning of the midpiece. The outer coarse fibers are thickest in this region and although they show some variation in shape, they are all nearly the same size.

Fig. 5. Transverse section of the principal piece showing that at this level, only seven of the nine dense outer fibers remain. Numbers 3 and 8 have already terminated. There are now very marked differences in the thickness of the fibers, with numbers 1, 5, and 6 much larger than the others. The longitudinal columns of the fibrous sheath (Lc) are approximately in the plane of the central pair of fibrils. The difference in density of the subfibers of each doublet is quite evident in this micrograph.
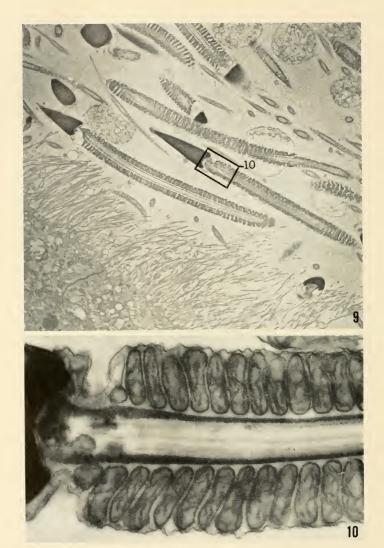
Fig 6. A section of guinea pig testis transecting three sperm tails at the level of the principal piece. The tapering inner edge of the longitudinal columns of the fibrous sheath project into the spaces occupied at a higher level by dense outer fibers 3 and 8, and thus divide the tail into two unequal compartments, one containing three and the other, four dense fibers. If the guinea pig sperm head is considered to be flattened dorsoventrally the longitudinal elements of the fibrous sheath are believed to run along the dorsal and ventral aspect of the tail, and the tail movements are believed to be mainly in the plane perpendicular to the line through the center pair of fibrils in the axial filament complex. (From Fawcett, 1961.) [*Note added in proof.* The direction of numbering of the fibers should proceed from fiber 1 in the direction toward which the "arms" on the doublets point. To follow this convention the position of numbers 5 and 6 should be reversed. D. W. F.]

mitochondria of the sperm middle piece coalesce late in spermiogenesis into a continuous strand wrapped spirally around the base of the flagellum, or whether they retain their individuality. It is now clear from the study of electron micrographs that either may occur. In insects, gastropods, and other invertebrates the conspicuous *nebenkern* formed during spermiogenesis arises by fusion of mitochondria. It subsequently spins out into a long mitochondrial strand that becomes intimately applied to the axial filament complex of the tail. In the vertebrates, and particularly in mammals, the mitochondria generally remain separate but there are marked species differences in their form and arrangement. In some species they are spherical, in others they are elongated and arranged end to end in a helical sheath around the fibrous core of the tail (Fig. 9). The proportions of the midpiece, the length of the individual mitochondria, and the pitch of the helix vary greatly from one mammalian species to another. The mitochondria may have the usual internal pattern of

FIG. 7. Section of guinea pig epididymis transecting a group of sperm tails at the level of the principal piece. Notice that the orientation of all the cross sections is the same. This is attributable to the fact that the broad flat sperm heads in this species stack up in rouleau in the third part of the epididymis. This brings all the tails of the same group into the same orientation. In this instance, presumably the flat heads would be stacked in parallel array with their long transverse axes perpendicular to the line (XY) through the central pair of fibrils. If we are correct in assuming that XY is the dorsoventral axis and that the bending movements are mainly transverse, then the tail is bilaterally asymmetrical, having three dense fibers on one side and four on the other. In the guinea pig and many other mammalian species the cross section of the tail is symmetrical with respect to the axis passing through fiber 1 and between 5 and 6, but in some species (see Fig. 8) it is impossible to find a plane of symmetry.

FIG. 8. Cross section through the midpiece of a bat spermatozoon. The mitochondria are flattened, semilunar in form, and of uniform size. There are two to each turn of the mitochondrial sheath and they meet end to end on or near the XY axis. This species is unusual in having four, instead of three, members of the outer row of dense fibers that are larger than the others and of very nearly the same size. These are numbers 1, 5, 6, and 9. No plane of bilateral symmetry can be drawn with this arrangement of fibers.

cristae (Figs. 10 and 11), or they may be extensively modified. The most dramatic example of the latter is seen in the opossum where there is a remarkable proliferation of mitochondrial membranes late in spermiogenesis that results in the formation of closely packed concentric lamellae that completely fill the interior of the organelle. As yet nothing is known about the changes in biochemical activities which may accompany this extreme modification of the internal structure of the mitochondria.

The mitochondrial sheath presumably participates in sperm locomotion mainly as a source of energy for the contraction of the longitudinal fibrils of the tail. If such is the case, one would expect that the energy requirements of two different kinds of sperm of roughly similar proportions would be much the same. The fact that sea urchin sperm or fish sperm have only a few mitochondria while mammalian sperm may have over a hundred has therefore been puzzling. Comparative electron microscopic studies to date suggest that those spermatozoa which have simple tails consisting only of the nine-plus-two complex of axial fibrils have a short midpiece made up of two to four mitochondria, whereas those spermatozoa which have an additional row of nine coarse fibers outside the regular axial filament complex have a long midpiece with a well-developed mitochondrial sheath. If the mitochondrial number is correlated with the energy needs of the cell, it would appear that the addition of an outer row of coarse fibers in the mammalian sperm has greatly increased the energy requirements for locomotion compared to that of spermatozoa propelled by simple flagella. This observation tends also to support the belief that the outer coarse fibers of mammalian

Fig. 9.   Low-power electron micrograph of bat epididymis. At the lower left is the free surface of the lining epithelium with its long stereocilia. In the lumen are a number of spermatozoa sectioned in various planes. Bat sperm have a long thick middle piece. An area like that enclosed by the rectangle is shown at higher magnification in Fig. 10. (Electron micrograph by Arthur Mitchell.)

Fig. 10.   The base of the sperm head is shown at the left and the connecting piece which is continuous with the dense outer longitudinal fibers of the middle piece. The mitochondria are flattened, very uniform in size, and closely applied to the outer dense fibers. The cristae in the mitochondria tend to run parallel to the limiting membrane.

11   12
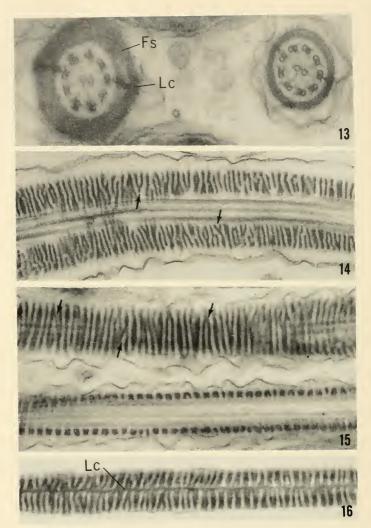
sperm tails are additional contractile elements and not merely passive supporting structures.

THE FIBROUS SHEATH OF THE PRINCIPAL PIECE

The presence of a fibrous sheath around the principal piece of the mammalian sperm tail was observed with the light microscope in the latter part of the nineteenth century (Jensen, 1887). It was depicted as a continuous fiber wound circumferentially around the axial filament. The early electron microscopic studies of intact sperm tails shadowed with metal perpetuated the idea that the sheath consisted of a single or double strand wound helically around the axial filament complex, and the terms *spiral sheath* or *tail helix* were often used to describe this component (Randall and Friedlaender, 1950). More recent electron microscopic studies based upon observations on thin sections have shown that this concept of the structure of the fibrous sheath is greatly oversimplified and largely erroneous (Fawcett, 1958).

In our opinion the sheath is not a continuous helical wrapping but is made up of a series of semicircular ribs which insert into two longitudinal columns that run along opposite sides of the tail (Figs. 5, 7, and 13). In cross sections of the principal piece of rodent sperm, conspicuous thickenings are observed on opposite sides of the sheath where the wider ends of the ribs merge with the longitudinal components of the sheath (Figs. 5–7). The thickenings of the sheath are always approximately in the plane of the central pair of fibrils, and the tapering inner edges of the longitudinal columns project inward toward fibers three and eight of the outer row. At levels distal to the

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Figs. 11 and 12.   Two bat sperm sectioned longitudinally through the base of the head, the neck, and anterior portion of the middle piece. The dense, homogeneous heads have been sectioned parallel to their long transverse diameter. The neck region contains a complex connecting piece that appears to be a greatly modified centriole and shows a periodic cross banded structure. Figure 11 is a median longitudinal section for most of its length. Figure 12 is more obliquely cut, so that the section in the lower half of the figure passes tangential to the mitochondrial sheath revealing that the end-to-end junctions of the mitochondria in successive turns of the sheath are aligned. Since these junctions were found in cross sections to be in the plane of the central pair of fibers, one can infer that that plane is perpendicular to the plane of flattening of the head.

162

termination of these two fibers the narrow edges of the columns project into the spaces which they formerly occupied, and come into close association with the corresponding doublets of the axial filament complex (Figs. 6 and 7).

It is only fair to say that this interpretation of the structure of the fibrous sheath is not yet universally accepted. Several investigators still deny the existence of any longitudinal components of the sheath and insist that the thickenings observed in cross sections are confined to the individual gyres of a helix (Schultz-Larsen, 1958; Ånberg, 1959). This position seems to us difficult to defend in the face of the evidence provided by longitudinal sections of sperm tails that pass nearly tangential to the sheath (Figs. 15 and 16). In such sections the successive circumferential elements of the sheath are seen to branch and anastomose frequently in a manner quite inconsistent with the interpretation of the sheath as a continuous helical wrapping. More-

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Fig 13.   Two bat sperm tails sectioned transversely at different levels in the principal piece. The circumferential elements of the fibrous sheath (Fs) are wide in the first part of the principal piece (illustrated by the section at the left), but become narrower toward the caudal end of this segment (as shown in the cross section at the right of the figure). The longitudinal columns of the sheath (Lc) are relatively inconspicuous in this species and appear in cross sections as slightly denser areas on opposite sides of the sheath.

Fig. 14.   A longitudinal section through the first part of the principal piece shows that the dense ribs of the sheath which appear broad in cross sections of the tail are actually quite thin. Some are interrupted, others branch (see at arrows) and connect with the next rib above or below. It is evident that the older interpretation of this sheath as a continuous fiber wound spirally around the longitudinal fibers of the tail was a great simplification.

Fig. 15.   Two adjacent sperm tails in the bat epididymis. The section has passed tangential to fibrous sheath of the uppermost sperm tail affording a surface view of the dense circumferential ribs. These can be seen to branch and anastomose (see arrows) in a manner that would not be expected of the successive gyres of a helix. The lower sperm tail in this figure is cut in mid-saggital section, at a level near the end of the principal piece where the ribs of the sheath are not so wide as they are in Fig. 14.

Fig. 16.   A section providing a surface view of the principal piece. This clearly shows the ribs joining the slender longitudinal column of the sheath (Lc). (From Fawcett, 1961.)

over, in subtangential longitudinal sections through one of the thickened parts of the sheath (Fig. 16), one can see very clearly where the ribs join a continuous longitudinal band. The size of this longitudinal component varies with the species but its reality is undeniable, even in the bat and in the human sperm tail where the thickenings of the fibrous sheath are much less conspicuous than they are in the rodents. In median longitudinal sections of the principal piece (Fig. 14), the ribs of the sheath are seen in cross section and display a pleomorphism entirely inconsistent with the older interpretation of the fibrous sheath as a simple helical wrapping.

Very little is known about the mode of formation, the chemical nature, or the functional significance of the fibrous sheath. Studies of its solubility properties indicate that it is a highly resistant scleroprotein (Bradfield, 1955). It is probably not directly concerned with producing the tail movements but may be an important supporting or elastic component of the tail. It has been suggested that it may have a springlike function, offering resistance to the waves of contraction that pass along the axial filament complex, thus providing the necessary couple for the transverse bending movements of the tail (Challice, 1953).

ORIENTATION OF TAIL COMPONENTS WITH RESPECT TO THE PLANE OF TAIL MOVEMENT

It has been established that cilia beat in a direction perpendicular to the line through the centers of the central pair of internal fibrils (Fawcett and Porter, 1954). Lack of fixed landmarks makes it more difficult to relate the internal structure of the sperm tail to the plane of its beat. In some species, however, the flattened shape of the head makes it possible to assign dorsoventral and transverse axes. From suitably oriented thin sections, one can then determine the position of middle-piece structures in relation to these axes. We have undertaken such a study in the bat sperm which has a structure that is particularly favorable for such an analysis.

The head of the bat sperm is somewhat flattened and the longer dimension of its elliptical cross section is designated the *transverse axis* and the shorter, the *dorsoventral axis*. The mitochondrial sheath is unusual in that the pitch of the helix is very flat and each gyre is made up of two crescentric mitochondria of equal size that meet on
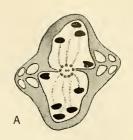
opposite sides of the fibrous core of the midpiece (Fig. 8). So uniform is the size of the mitochondria and so regular is their arrangement that their end-to-end junctions in the successive gyres form two straight lines on opposite sides of the middle piece. Moreover, it can be shown in transverse sections of this segment that the lines of end-to-end junction of the mitochondria nearly coincide with the plane of the central pair of fibrils in the axial filament complex (Fig. 8). The line of mitochondrial junctions in the midpiece of the bat sperm therefore constitutes a surface landmark which is a useful guide to the orientation of the internal fibrils. Thus in oblique sections that pass through the head parallel to its transverse axis and include the first part of the midpiece (Fig. 12), it can be ascertained that the line of junctions of the mitochondria (and also the central pair of fibrils) is in the dorsoventral axis of the spermatozoon. If it be assumed that the plane of bending movements of the tail is at right angles to the plane of the central pair of fibrils, as is true of cilia, then it can be concluded that the tail movements in this species are mainly in the transverse plane. The same may prove to be true of other mammalian sperm whose heads are flattened dorsoventrally. Similar observations on oblique sections at the junction of the middle piece and principal piece reveal that the opposite longitudinal components of the fibrous sheath are on or near the dorsoventral axis of the spermatozoon.
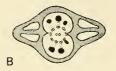
When fresh bat sperm are examined with the phase contrast microscope, it is possible, where sperm heads present their broad face, to make out a thin luminous line along the middle piece which apparently represents the line of junction of the mitochondria. The presence of a visible surface landmark in the middle piece that is related to the orientation of the internal fibrils may make bat sperm particularly favorable material for future studies correlating cinematographic analysis of the tail movements with the fine structure and arrangement of their internal fibrous components.

CONCLUDING COMMENT

The objective of comparative electron microscopic studies of spermatozoa is to discover which structural features are common to all mammalian species, and which are peculiar to one or to only a few.

It is a basic assumption of this approach that those components and spatial relations which are of universal occurrence are essential to motility, and those that are of more restricted occurrence are likely to be merely variations related to the internal mechanics or the peculiar locomotor problems of a particular species. The axial fila ment complex is characterized by a precise uniformity in the size and position of its eleven fibrils, whereas in the outer coarse fibers of the sperm tail, variation in length and diameter appears to be the rule. This variation is not random however. Three large fibers, num bers 1, 5, and 6, are found in rat, guinea pig, cat, and man, and at first it seemed likely that this pattern of size variation would prove to be of general occurrence among mammals. Exceptions are now beginning to appear. In the bat, for example, there are regularly four of the dense outer fibers that are larger than the other five. These are numbers 1, 9, 5, and 6 (Fig. 8).

It is evident from examination of cross sections of sperm tails of several species that although the basic organization of the fibrous sheath is much the same, there are marked differences in its thick ness and in the cross-sectional profile of the tail (Fig. 17). The inter esting suggestion of Cleland and Rothschild (1959) based upon study of the bandicoot (Fig. 17A), to the effect that the distance of the outer coarse fibers from the axial filament complex is related to the thick ness and stiffness of the sheath, is apparently borne out in the opos sum (Fig. 17B) where certain of the fibers of the outer row are placed some distance away from the corresponding member of the inner row. In rat and guinea pig (Fig. 17C and D) it might be argued that the relatively greater diameter of fibers 1, 5, and 6 may overcome the resistance of the sheath to bending, without moving the fibers farther away from the hinge point. Examination of a cross section of the bat sperm tail (Fig. 17E), however, leaves us in some doubt concerning the validity of this line of reasoning. Here, despite an unusually thick middle piece, and a very thick fibrous sheath in the principal piece, the peripheral dense fibers are relatively slender, short, and closely applied to the axial filament complex. It is clear that we still have much to learn about the outer longitudinal fibers and the cir cumferential elements of the fibrous sheath and about their respec tive roles in sperm locomotion. Further comparative observations may provide clues to their function but ultimately ways will have
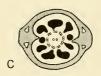
Fig. 17. Diagrammatic cross sections of the principal piece of the sperm tail in several different species, showing the variations in the shape and thickness of the fibrous sheath and in the size and position of the outer dense fibers. A. Bandicoot (from Cleland and Rothschild, 1959). B. Opossum C. Rat. D. Guinea pig. E. Bat.

to be found for dissociating the inner and outer longitudinal fibers and the fibrous sheath for separate analysis of their chemical composition and physical properties.

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Cytochemical Aspects of Spermatozoan Motility*

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The current symposium is concerned with the inherent motility of sperm, in spite of recurrent reports that in this or that organism, live and dead sperm may with equal facility be transported from the site of deposition to the site of ovum activation in the female reproductive tract. The reassuring conclusions of Noyes and co-workers (1958), based on observations that neither semen nor radio-opaque fluids move through the rabbit cervix during female orgasm, attest to the fact that the motility of the sperm themselves is responsible for their passage in this animal at least through the cervical barrier.

This paper will concern itself with the problem of what may be inferred concerning motility mechanisms from chemomorphological considerations. Much information may be gleaned from studies of ultrastructure in defining a common denominator among the organelles found in cilia and flagella. However, in light of the wide diversity both of biochemical attributes and of gross and fine structure, generalization on the common character of ciliary and flagellar action, on that of flagellates and spermatozoa, or even on that of mammalian and invertebrate sperm may not yet be warranted. The recent electron micrographs of Fawcett (this symposium), Cleland and Rothschild (1959), Afzelius (1959), and of Gibbons and Grimstone (1960), among many others, merit the seal of approval as standards of elegance in depicting the flagellum in all its geometrical beauty, revealing the fine structure to critical scrutiny. The flagellum has been admirably and exhaustively described in other papers, but

I should like briefly to outline a frame of reference on which to hang some remarks. The sperm, once launched, may be regarded as a self-propelled device perhaps endowed with a guidance system sensitive to external signals, such as sperm-egg interacting substances. It is powered by fuel which may be continuously resynthesized at or near the site of utilization, either from stored precursors or from substances which diffuse or are actively transported across the cell membrane. Energy for the synthetic reactions may be derived from fructolysis or glycolysis or by oxidation of substances such as the products of phospholipid metabolism (Hartree and Mann, 1959). (There is no evidence at hand that fuel may be delivered by trickling through the apparently undifferentiated matrix of the flagellum or through "hollow" longitudinal tubules from fuel generators in the midpiece.) Initiation of the propulsive wave may be triggered by dilution with suitable media, namely seminal plasma constituents or other natural or synthetic physiological solutions, which provide an adequate stimulus even in the absence of extraneous supplemental substrate. The coordination essential for effective propulsion has been postulated by Bradfield (1955) as the function of a conductile system, possibly the central pair of longitudinal fibers or the nine axial fibers (all of which coincidentally have different solubility characteristics from the nine peripheral fibers of the mammalian sperm). Once the wave has been initiated in a given peripheral fiber, it continues as a succession of propagated localized contractions, and if each of the nine peripheral fibers is contractile, the neighboring fibers sequentially proceed to contract in coordinated fashion in a particular phasic relationship. A cycle of alternating contraction and relaxation phenomena may serve intrinsically to regulate the propagation of the undulatory wave.

I should like to consider in detail some of these attributes of the moving mammalian spermatozoon in the context of experimental procedures designed to relate chemical events to specific morphological characters. The presence of both adenosine triphosphate (ATP) and adenosine triphosphatase (ATPase) in sperm has been known since the work of Lardy and colleagues (1945), Mann (1945), Rothschild and Mann (1950), and Engelhardt (1946). During the last decade, evidence was obtained showing not only the ATPase (Nelson, 1954; Tibbs, 1957) but also succinic dehydrogenase and cytochrome

oxidase (Nelson, 1955) to be concentrated in almost equal proportions in the midpiece and tail (Fig. 1). This does no violence to Gray's postulate (1928, p. 49) that a "moving wave cannot provide energy for propelling an organism and at the same time pass on with unreduced amplitude unless the energy being lost is continually being replaced as the waves move along." In a theoretical analysis of wave propagation along flagella, Machin (1958) has demonstrated that some of the energy fed into each element of the flagellum will be used to overcome viscous forces of the medium and that the remainder of the energy is absorbed in elastic deformation of the element. Unless a reversible mechanism exists to convert the elastic energy back into chemical energy, it is irrecoverable and will degrade into heat in the elastic or contractile components.

The application of cytochemical techniques to frozen-dried material subsequently processed for electron microscopy has now made it possible to substantiate, with a somewhat higher degree of resolution, the data obtained from isolated sperm fragments. The coincidence of distribution of enzymes concerned with the utilization and resynthesis of ATP establishes the rationale for ascribing the generation of the undulatory wave to the flagellum itself (Nelson, 1958a, 1959b) (see Figs. 2 and 3). Enough ATP may be resynthesized via oxidative phosphorylations at the disposal of the flagellum (Nelson, 1958b) so that the mechanical reconversion of the energy of deformation into chemical energy need not be limiting (even when the power expended amounts to 2×10^{-7} erg/sec, as noted by Rothschild at this symposium).

If the peripheral fibers are contractile structures responsible for propulsion of the sperm, then, according to Cleland and Rothschild (1959), they may be regarded as tensile elements; parts of the fibrous "spiral" sheath may consist of compression elements which become deformed as the peripheral fibers contract. Electron micrographs (Fig. 4) of frozen-dried rat sperm may offer a clue to the site and nature of the deformations. Since, of course, the peripheral fibers themselves are deformed in the course of their undulations, Machin (1958) proposes that the amplitude of the wave could be sustained by energy arising in the more distal elements, activated as a result of the "passively propagated" bending wave. This limitation of the "actively induced bending" to the proximal end of the flagellum would
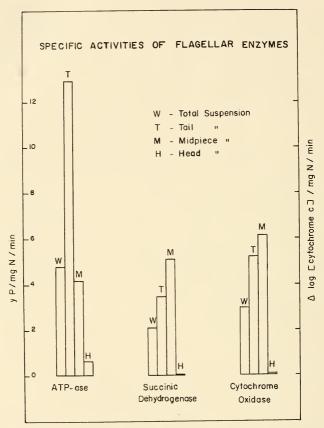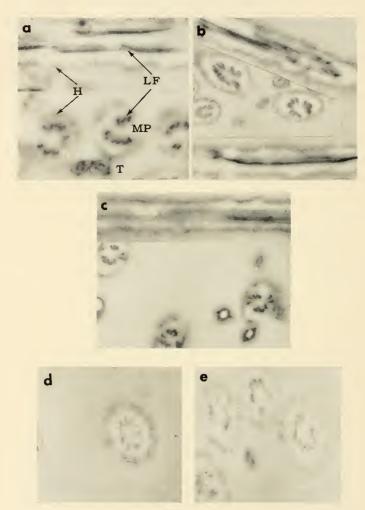
FIG. 1. Specific activities of bull sperm enzymes. ATPase, micrograms phosphorus liberated from ATP per milligram of nitrogen (of suspension) per minute. Succinic dehydrogenase, Δ log [Ferricytochrome c] per milligram nitrogen per minute. Cytochrome oxidase, Δ log [Ferrocytochrome c] per milligram nitrogen per minute. Enzyme activity is concentrated in tail and midpiece fraction, negligible activity in head fraction shown for comparison. Both reduction and oxidation of cytochrome c are relatively uniform throughout flagellum.

FIG. 2. Adenosinetriphosphatase in frozen-dried rat sperm. (a) Transverse and (b) longitudinal sections of epididymal sperm incubated in ATP, $CaCl_2$, cysteine, veronal buffer pH 9. The peripheral fibers are densely "stained" compared to sheath, axial core, and matrix. Controls: (c) ATP-free medium; (d) $CaCl_2$-free medium ($\times 9,000$).
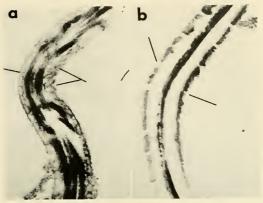
Fig. 4.   Distortion of sheath elements during bending of flagellum. Frozen-dried rat sperm extracted for 10 min in $0.6M$ KI after glycerination. Stained with $PtBr_4$. Longitudinal sections of (a) midpiece and (b) tail. Note reorientation of sheath elements on concave surface of midpiece compared to those on convex surface; on the convex surface of the tail there is an apparent separation of the sheath elements and a compression of these elements on the concave surface ($\times 20,000$).

further obviate the necessity for a mechanism to transmit control information to the contractile elements. It would be difficult to reconcile this argument with the fact that, in rat epididymal sperm, the region anterior to the still unshed kinoplasmic droplet appears quiescent and relatively rigid, while the region distal to the droplet thrashes about actively (Nelson, 1959b). Not only does this observation imply that at least a large proportion of the flagellum, under appropriate conditions, possesses the capacity to participate in localized contractile activity, but also that a transmission system inde-

Fig. 3.   Succinic dehydrogenase in frozen-dried rat sperm. Longitudinal and transverse sections incubated in (a) polymeric nitro BT tetrazolium and succinate, (b) p-nitrotetrazolium and succinate, (c) K-tellurite and succinate, (d) veronal buffer and succinate—a control, (e) buffer alone—another control. H, helical sheath; LF, peripheral fibers; MP, middlepiece; T, tail. Note that peripheral fibers are of higher electron density than helical fibrous sheath in tetrazolium- and tellurite-treated specimens, while there is no such density differential in the controls ($\times 18,000$).

pendent of, but concerned with the control of, the contractile ele-
ments may be built into the flagellum. Tibbs' report (1960) that
fresh-water fish sperm heads contain acetylcholinesterase confirms
that of Sekine (1951), who stated that the sperm enzyme was nearly
as active as that of brain tissue. It may be fortuitous that the histo-
chemical incubation medium which contains a high concentration
of Na_2SO_4 to prevent diffusion of the cholinesterase appears to pre-
serve the axial core of the frozen-dried rat sperm flagellum which in
other media is quite labile. In fact, preliminary experiments which
I have been undertaking indicate that at least part of the axial core
may prove to contain cholinesterase activity. While this is at variance
with Tibbs' findings in trout and perch sperm, it is in agreement
with Bradfield's prediction (1955) concerning the locus of a con-
ductile apparatus. A propos of the question of coordination and the
allied problem of guidance, Rothschild (1956) inquired several years
ago whether waves which travel along the tails can be bilaterally
asymmetrical when necessary. The consensus has been that periph-
eral fibers 1, 5, and 6 of mammalian sperm effectively determine the
plane of flagellation by virtue of their large size and their orientation
with respect to the plane of symmetry which includes the central
pair of longitudinal fibers (Fig. 5). Inasmuch as the other six fibers
(2, 3, 4, 7, 8, and 9) are also cytochemically positive for ATPase, in
spite of their less favored position vis-à-vis the "plane" of flagellation,
they could presumably act in the guidance of the sperm by contrib-
uting to the asymmetrical deformation of the flagellum (unless each
of the nine peripheral fibers is indeed sequentially involved in the
propagated undulations).

Plasticity of the flagellum is undoubtedly a necessary condition
for wave generation. When ATP is depleted or washed out, flagella
frequently appear straightened or rigid in a manner akin to muscle
rigor. It is conceivable that in the undulating spermatozoon, the
passage of the undulatory wave temporarily and locally diminishes
ATP below a critical level so that a relaxation factor may be required
to intervene to maintain the plasticity during regeneration of the
ATP (Nelson, 1959a). The search for relaxing factors in muscle has
turned up a number of substances which behave as plasticizing agents,
including excess ATP, ATP-rephosphorylating systems (phosphoenol-
pyruvate or creatinephosphokinase), chelating agents, ATPase in-

hibitors, and inorganic pyrophosphate. Of particular interest is the last-named substance; sodium pyrophosphate (NaPP) delays the dilution-precipitation of washed suspension of *Mytilus* sperm tails, while ATP has an accelerating effect. Moreover, among other enzymes found in these isolated flagella is a highly active inorganic pyrophosphatase (IPPase), the substrate of which, NaPP, in appropriate concentrations inhibits the ATPase and competes with the flagellar ATPase for Mg (Nelson, 1959a). This enzyme, also concentrated in the peripheral fibers of mammalian sperm flagella (Fig. 6), is soluble in 50% glycerol, whereas the ATPase is stable (Nelson, 1959c), and remains *in situ*. One possible interpretation of the repetitive twitch behavior of Bishop's glycerinated sperm models (Bishop, 1958a,b) contrasted to the oscillations of fresh sperm is that progressive movement requires the reciprocal interaction of at least two
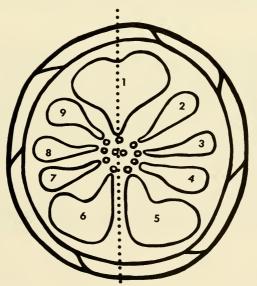
Fig. 5. Diagram of transverse section of rat sperm midpiece. Dotted line through central pair of fibers indicates plane of symmetry and possibly of undulation.
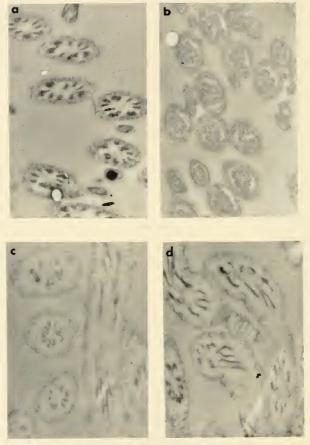
Fig. 6. Inorganic pyrophosphatase in frozen-dried rat sperm (a) incubated in Na-pyrophosphate 12 min; (b) control—no substrate; (c) incubated in Na-pyrophosphate, 30 min, after glycerination at −20°C for 4 weeks; (d) control—glycerinated 4 weeks. Note that enzyme has been extracted by prolonged treatment with 50% glycerol (×15,000).

enzyme systems, one of which is inactivated or washed out during glycerol extraction. The presence of glycerol-soluble IPPase suggests, as a mechanism of motility, the cyclic interaction of this enzyme with ATPase. The ATP-Mg-ATPase system dominates the contraction phase while something like the NaPP-Mg-IPPase system could participate in the relaxation phase (Nelson, 1959a).

With respect to the contraction phase of sperm movement, electron dense "bridges" extending from the peripheral fibers to the axial fibers may tempt one to consider a Huxley-Hanson type of sliding filament mechanism (Fig. 7) in favor of a model based on molecular folding within the peripheral fibers themselves (Nelson, 1958b). The analogy suffers as yet from the lack of evidence for any clear-cut
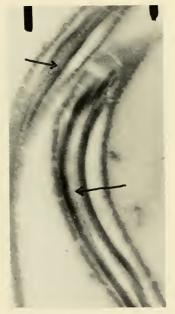
FIG. 7. Projections extending between peripheral fibers and axial fibers, indicated by arrows. Note similarity to bridges between actin and myosin filaments of Huxley-Hanson muscle preparations ($\times 40,000$).

"interdigitation" and sliding, although the two sets of fibers involved unquestionably differ in their solubility characteristics. Muscle specialists caution that even though a motile system possesses contractile elements which exhibit ATPase activity, this evidence alone does not justify the inference that the system is a muscle. Nevertheless, the judicious application of muscle methodology to sperm investigations may yield constructive information. For example, Burnasheva (1958), after treating bull sperm with known actomyosin extractants obtained "spermosin," a substance which on the basis of its reactions with actin and ATP would be classified as a myosin-like protein (Fig. 8). In fact, myosin antibodies react to some degree with rat sperm at the electron microscope level. Extraction of the frozen-dried sperm with 50% glycerol does not appreciably diminish this reactivity, while subsequent treatment with the actomyosin extractants abol-
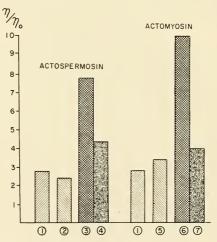
FIG. 8. Viscosity changes with complex formation and addition of ATP in system composed of extracted bull sperm protein and rabbit muscle actin; analogous reactions of actomyosin shown at right. 1, F-actin; 2, spermosin; 3, spermosin + F-actin (ratio 12:1); 4, spermosin + F-actin + ATP ($4.23 \times 10^{-4}M$); 5, myosin; 6, myosin + F-actin (ratio 2:1); 7, myosin + F-actin + ATP ($4.23 \times 10^{-4}M$). (Redrawn from Burnasheva, 1958.)

FIG. 9. Myosin antibody reactivity of frozen-dried rat sperm. (a) Incubated in myosin antibody preparation 30 min; (b) control in normal rabbit serum. (Not illustrated are specimens incubated in antibodies and normal serum preparation after prolonged extraction with 0.6M KI and other actomyosin and myosin extractants; after such treatment, there is no longer any localized increase in electron opacity due to antibody adsorption.) (×15,000.)

ishes the reactivity (Nelson, 1961) (Fig. 9). However, if similarly extracted sperm are stained with PtBr$_4$, the peripheral fibers may still be seen, confirming the complex nature of the fibers suggested by the previously mentioned investigations in sperm cytochemistry. Burnasheva's report (1958) that extracted spermosin complexes with muscle actin implies that spermosin more nearly resembles myosin than actomyosin and that therefore sperm actually lack actin. While

Vorobiev (1957) and Ashmarin (1953) claim that myosin *in vitro* complexes with other polyelectrolytes besides actin to produce contractile threads, this does not warrant the conclusions that actin or an actin-like protein is absent from sperm. Hayashi *et al.* (1958) have shown that finite quantities of actin are required to confer contractility on purified myosin; and Pautard (this symposium) claims to have found actin in fish sperm. Perhaps the hypothetical actin homolog of mammalian sperm resists extraction by methods suitable for its removal from muscle. Alternatively, the other-protein interaction in mammalian sperm may be of such a nature that the spermosin is freely extractable, leaving the actin-like moiety bound to a different flagellar component. Should this be the case, the binding sites on spermosin *in situ* could perhaps be accessible to the non-flagellar actin. Preliminary tests, in which frozen-dried rat sperm were incubated in purified G-actin solutions, give the distinct impression that the muscle actin is adsorbed on the surface of the peripheral fibers and fills the matrix between the fibers, while bovine serum albumin of approximately the same concentration (0.1%) does not appear to combine with any flagellar structure. Prior extraction of the frozen-dried sperm with $0.6M$ KI in $Na_2S_2O_3$ interferes with the increase in electron density attributed to the actin adsorption (Fig. 10).

It may be premature at this juncture to speculate on the actin-spermosin interaction in terms of complementarity or other type of macromolecular affinities. The time scale imposed by the contraction-relaxation cycles necessitates the formation of a freely dissociable complex. The logical site for the actin-like member of such a complex would be within the axial fiber system, although Varga and co-workers (1955) associate the L-meromyosin fraction of skeletal muscle with acetylcholinesterase (assuming the acetylcholinesterase of mammalian sperm to be within the axial core). More direct evidence may have to await the application of purified actin antibodies in the cytochemical procedures, in conjunction with prolonged extractions in high ionic strength media.

What may we conclude from the present cytochemical considerations that may lend substance to our speculations on the morphological basis of sperm motility? Some of the necessary ingredients for the generation and regulation of the undulatory wave have been identi-
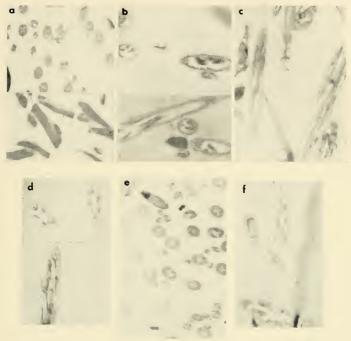
FIG. 10. Adsorption of purified actin by components of frozen-dried sperm. (*a*) Transverse and longitudinal sections of sperm exposed to G-actin, 45 min; (*b*) similar preparation in bovine serum albumin; (*c*) soaked in deionized water 45 min. After 50 min extraction with 0.6M KI in 0.06M Na$_2$S$_2$O$_3$; specimens were treated as previously: (*d*) G-actin; (*e*) bovine serum albumin; (*f*) deionized water ($\times$6,000).

fied. Moreover, they have been fitted into the organization of the flagellum. The peripheral fibers which contain ATPase, succinic dehydrogenase and inorganic pyrophosphatase are also reactive with myosin antibodies. Actomyosin extractants abolish reactivity with the antibodies, at the same time removing substances which adsorb extraneous actin. Glycerination washes out IPPase without affecting ATPase or antibody reactivity. Alkaline phosphatase, which may be

concerned with the transport of substances across cell membranes, appears to be localized in the "spiral" sheath confining the flagellum (Nelson, 1958a). The axial core is stabilized by cytochemical reagents used in the demonstration of cholinesterase, and is a logical contender for the actin-like moiety of the contractile complex. Projections from the peripheral fibers extend inward toward the axial fibers in a manner reminiscent of the Huxley-Hanson sliding filament pattern. However, while only 3–5% localized shortening may be required for the propagation of the sinusoidal wave, we may still have to invoke molecular folding of the "actinoido-spermosin" complex. Even though all the loci could not have been anticipated in detail, the results thus far permit the construction of an effective, yet sufficiently flexible framework for deferred incorporation of unassigned components and for verification of tentative speculations.

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Biomolecular Aspects of Spermatozoan Motility

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Since the advent of the electron microscope and the observations of Manton and Clarke (1952) the universal character of the flagellum and cilium based on a system of nine outer and two inner fibrils has been well established, and there is an increasing body of literature listing the detailed cytology of this arrangement (Bradfield, 1955; Fawcett and Porter, 1954; Afzelius, 1959; Gibbons and Grimstone, 1960). On the other hand, although the biochemistry of spermatozoa (Mann, 1954, for example) and the separated flagella from spermatozoa (Nelson, 1954; Tibbs, 1957) have been studied, the contractile nature of the "9 plus 2" flagellum in relation to other forms of motility still remains obscure. In any study of spermatozoan movement there seem to be three questions which need to be answered. First, is the flagellum a self-contained contractile unit independent of the cell? Secondly, what is the nature of the contractile substance and does it function in the same way as in other motile systems? Lastly, if there is some common plan of movement throughout nature, what is it?

The following account is an attempt to answer these questions and is based on a study, spread over several years, of those 9 plus 2 flagella which seem to offer the best opportunities for separation and analysis of the contractile material.

CHOICE OF SUBJECTS

Biomolecular studies, based on x-ray diffraction, infrared absorption, and similar techniques, require relatively large quantities of material, and this factor has restricted, for the present, the kinds of flagella that can be considered. Also, it was found that the cilia from initially promising subjects like *Vorticella, Carchesium polypinum,*

189

Stentor coeruleus, and *Spirostomum ambiguum,* and the flagella from unicellular algae (such as *Polytoma uvella, Polytomella caeca,* and *Chlorogonium elongatum,* which can be grown under controlled and sterile conditions to yield useful quantities of flagella) could not be separated clearly from the cells without rupture of the cell wall and extrusion of cytoplasm.

Spermatozoa appeared to be the remaining subjects most likely to provide pure preparations of flagella, but most of these were rejected either because the flagellum was too complex to provide a fraction reliably identifiable as the "9 plus 2" system, or because the supply was erratic. Fish sperm were by far the most profitable source of flagella, but of those considered, only two fresh-water fish, the brown trout (*Salmo fario*) and the perch (*Perca fluviatilis*), were available locally and in sufficiently large quantities to permit adequate study. The sperm from these two fish (Fig. 1) have the great advantages of simplicity and length of flagellum, apparent absence of a midpiece and other mitochondrial apparatus, comparatively reliable separation of tails from heads, and high yield of flagellar substance. An average preparation of 100 mg of dry flagella was obtained from fifty ripe trout, but unfortunately this otherwise ideal source of flagella was limited by the shortness of the spawning season, so that research, at least on fresh material, was confined to about three months of the year.

EXPERIMENTAL PROCEDURE

The culture of the unicellular algae *Polytoma uvella, Polytomella caeca,* and *Chlorogonium elongatum* and the separation of flagella have been described elsewhere (Pautard, 1958a) and will not be considered here.

Fish sperm were obtained from a number of sources. Small supplies of cod (*Gadus morrhua*) sperm, removed from whole chilled testes, were sent from the Torry Research Station, Aberdeen, and similar quantities of cooled herring (*Clupea harengus*) sperm were supplied from the White Fish Authority laboratory at Lowestoft. Fresh-water fish, the main source of spermatozoa, were either kept in tanks until required or the milt was dispatched in sealed bottles from a local supply. During the latter part of April and early May, quantities of ripe male perch were supplied by the Biological Research Station, Lake Winder-

FIG. 1.   Trout and perch spermatozoa. (a) Electron micrograph of trout spermatozoon. Fixed in osmic acid vapor after suspension in 0.03M KCl buffered with phosphate to pH 6.8. Unshadowed (×3600). (b) Electron micrograph of perch spermatozoa. Fixed in formaldehyde in the milt. Gold-palladium shadowed (×4500).

mere, Westmorland. In November and December, the spawning months for the brown trout, ripe fish and regular supplies of milt were made freely available by the Welham Park Fish Hatchery, Malton, Yorkshire. From time to time, other fresh-water fish, such as the pike (*Esox lucius*) and the char (*Salvelinus willoughbii*), became available.

Ripe fish were stripped of milt by carefully wiping dry the skin surrounding the urogenital papilla and cloaca and then expressing the milt by gentle pressure along the line of the testes. Drops of water accidentally introduced into the milt tended to produce motility in the sperm, while fecal contamination usually resulted in the cells becoming inactive. Both these hazards were avoided as much as possible, and the viability of the sperm was checked at frequent intervals under the optical microscope.

Several procedures were adopted for the separation of tails from heads. In the case of the unicellular algae, careful shaking was essential to keep cell rupture down to a minimum, but in fish sperm the heads seemed to remain intact even after vigorous shaking, although the flagella themselves were disorganized depending on the way in which they were prepared. In earlier preparations, the spermatozoa were either allowed to swim in distilled water or immobilized by diluting the milt with phosphate buffers ($I = 0.17$) at pH 6.5. The suspended cells were then usually centrifuged at about $3000 \times g$ for 15 min, redispersed in a suitable (about 50 vol) amount of distilled water and shaken sharply for a minute or so. The heads were separated from the tails by several spinnings of the supernatant (containing the lighter tail fraction) at $3000 \times g$ for periods up to 15 min. When the supernatant was free of heads, the flagella were sedimented at about $10,000 \times g$ for 10 min and washed once or twice with distilled water by dispersion in suitable volumes of liquid, followed by centrifugation. Flagella prepared in this way (Fig. 2a) were usually frayed and fragmented but nevertheless useful for extraction.

Later, after it was found that distilled water removed into solution a proportion of the tail material, 0.03M KCl, buffered to pH 6.8 with phosphates (0.01M K$_2$HPO$_4$, 0.005M KH$_2$PO$_4$), was used to stabilize the flagella. In these preparations the milt was first centrifuged at $10,000 \times g$ for 5 min and the supernatant seminal fluid was carefully removed with a pipet. The densely packed cells were then suspended in 20 vol cold buffered KCl solution (the sperm were not motile) and
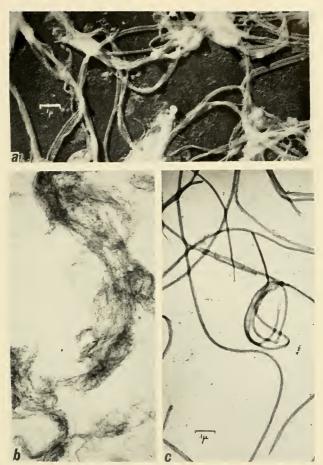
FIG. 2. Preparation of fish sperm flagella. (a) Electron micrograph of perch sperm flagella. Preparation washed in distilled water and fixed in formaldehyde. Gold-palladium shadowed (×6500). (b) Optical micrograph of trout sperm flagella prepared in 0.03M KCl buffered to pH 6.8 with phosphate (0.01M K_2HPO_4, 0.005M KH_2PO_4). In buffer solution, stained with gentian violet (×400). (c) Electron micrograph of the same preparation as (b). Fixed in osmic acid vapor before washing with distilled water. Unshadowed (×6500).

193

shaken vigorously in a tall, stoppered cylinder. Centrifugal classification followed as before without washing the finally sedimented flagella with distilled water, although they were sometimes rinsed briefly with buffered KCl. Unlike previous preparations, these flagella, illustrated in the optical micrograph in Fig. 2b and the electron micrograph in Fig. 2c, were mostly intact, with very little contamination by small fragments or any appreciable quantity of membranes from the head. Moreover, suspensions of these tails in salt solutions showed a marked streaming birefringence.

For x-ray diffraction studies, the flagella were suspended in distilled water or salt solutions and dried down on glass blocks treated with a water repellent. In earlier experiments, the salts left in the preparation were removed after drying or, more often, the flagella were washed with distilled water before the final suspension. Later, when buffered salt solutions were used, the flagella were commonly dialyzed until salt-free, and nondialyzable but soluble matter was dried down as well. Films cast from these dispersions and solutions were cut into strips about 1 mm across and mounted one on top of the other, usually on stretching frames. The stacks of films were stretched in damp or saturated air and frequently examined under moist conditions. Diffraction patterns were usually taken at 4 cm specimen-to-film distance in a flat film camera using nickel-filtered CuK α radiation collimated by glass capillary.

Specimens for infrared analysis (kindly undertaken by Dr. K. D. Parker) were cast as very thin films on glass and stripped as required, or cast and retained on thin, infrared transparent sheets of polythene.

GENERAL OBSERVATIONS ON THE MOTILITY OF FISH SPERMATOZOA

In those sperm that have been studied, movement is of short duration. There appears to be little motion of the cells in seminal plasma, but on dilution with water, the sperm disperse with a characteristic writhing and billowing of the milt. This intense activity lasts for a matter of seconds only. Individual cells can be seen moving in a straight line before they cease swimming and settle down to a brief period of oscillation. The active swimming phase lasts for about 15 sec in the trout, 12 sec in the perch, and 10 sec in the char, but the subsequent oscillatory behavior persists for 2 or 3 min and sometimes longer in in-

dividual cells. However, there is considerable variation in the activity of different spermatozoa. In some cases, the swimming stage is replaced by a longer period of oscillation—up to about 15 min. Inactive milt, from unripe fish or as a result of contamination, usually contracted when diluted with water, but this "inactive" material could usually be extended or dispersed by the addition of NaCl, $CaCl_2$ at pH 9.0, or Mg^{++} at neutrality. Although the sperm in these experiments tended to disperse as if they were viable, they did not, with few exceptions, swim or oscillate. After storage of viable milt, on the other hand, sperm activity diminished or ceased but could be restored, partly or completely, by the addition of electrolytes, particularly Ca^{++}, Mg^{++}, Na^+, and Cl^- at an optimum pH of 8.5. K^+ conferred only feeble activity, and NH_4^+ none at all. Some characteristic effects of ions on the restoration of activity of these enfeebled sperm are shown in the graphs in Fig. 3a–d. Among the substances conferring highest activity, the most surprising was bone dust, which apparently has no definable solubility product (Hodge, 1955).

In all these experiments on sperm from about 1000 fish of the species described, ATP (as a $0.003M$ solution at pH 7.0 with added $0.005M$ $MgCl_2$) did not have any effect on cell motility. On the other hand, spermatozoa that had actively swum and had ceased to move could be partially restored to motion by the addition of ATP. In some cases, isolated cells were seen still oscillating several hours later.

These results illustrate the dependence of this type of flagellum on the surrounding medium. Increased mobility at pH above 7.0 has been observed in many cells, although the maxima differ (Lardy and Phillips, 1943). The effects of NaCl on sperm movement are well known, but the relative effects of combinations of ions at different pH values seem to have received less attention. Unlike mammalian sperm, fish sperm have a short life in an environment that cannot be controlled by the fish, so that the cell, and perhaps the flagellum alone, must be "primed" for activity. Viable sperm can swim in water free of electrolytes, but once enfeebled or disturbed in any way, the cell cannot make good the deficiency, and the necessary stimulus and support must come from outside. Of all flagellated organisms and gametes, perhaps the fish spermatozoon is least fitted for controlled and sustained movement and may represent a much simpler arrangement than is found elsewhere.
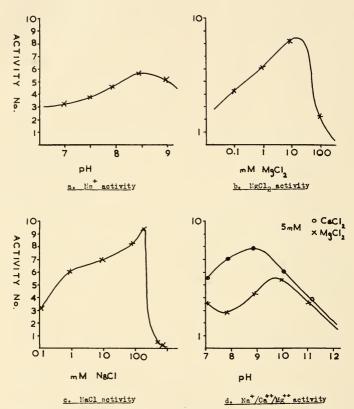
FIG. 3. Effect of electrolytes in restoring the activity of enfeebled trout sperm. Graphical interpretation is the mean of several observations. The activity number is an arbitrary scale varying from full motility (10) through oscillation of all cells (6) to one cell seen oscillating in every field at high power (1) to no movement whatsoever (0).

X-RAY DIFFRACTION

X-ray diffraction studies of biological subjects can often provide most fundamental indications of their nature. The muscle proteins, for example, are a remarkable illustration of Astbury's x-ray classification of the configurations of the fibrous proteins (see for example Astbury, 1947a). Muscle gives a wide-angle pattern typical of the α-configuration common to all proteins of the k-m-e-f (keratin-myosin-epidermin-fibrinogen) group. There is, besides, a number of meridional reflections identifiable with actin (Astbury et al., 1947), a globular protein with apparently close molecular-biological affinity to feather keratin (Astbury, 1947b). This combination of two proteins represents a "pair-in-hand" arrangement that might be common throughout nature, with one or the other partner predominating in a particular tissue, some tissues, however, such as porcupine quill and reptile keratin, suggesting intermediate states. In this scheme of things, muscle is simply part of a much wider plan, but it does show, perhaps more clearly than any other tissue, how closely the two molecular species are coupled together in the k-m-e-f group of proteins. In the bacterial flagella, x-ray diffraction studies (Astbury et al., 1955) have shown that even here, too, in this simplest of contractile organelles, the same plan is present and the same combination of actin-like and myosin-like molecular species occurs. Moreover, the diffraction pattern of bacterial flagella shows an added arc on the meridian at 4.65 A, the cross-β reflection (Rudall, 1946) being attributed to the folding of polypeptide chains transverse to the fiber axis. This configuration is closely associated with the supercontracted state (see, for example, Astbury et al., 1959, for details and history) that can be induced in all the k-m-e-f proteins, and the property of shortening from the folded α-form to the collapsed cross-β form has been proposed by Astbury as the ultimate molecular basis of muscular contraction (e.g., Astbury, 1947a).

In the flagella based on the 9 plus 2 system there is no report of similarities in detail with the x-ray diffraction patterns from muscle and bacterial flagella. Salmon sperm tails, separated by ultrasonic methods, give a diffuse protein pattern (Lowman and Jenson, 1952), while the diffraction pattern of algal flagella reported by Astbury and Saha (1953) is now seen to arise from an artifact (Astbury and Pautard,
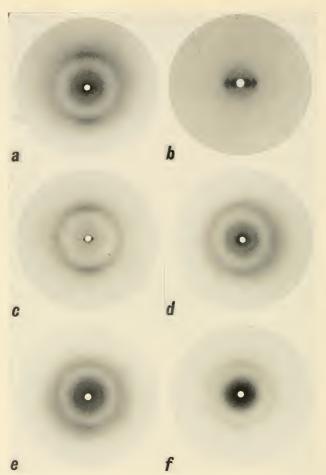
Fig. 4.   X-ray diffraction patterns from flagella. (a) Trout sperm flagella, stretched by about 150% in moist air. X-ray diffraction diagram at 4 cm specimen-to-film distance. (b) Perch sperm flagella, stretched by about 100% in moist air. Low-angle diagram at 6 cm specimen-to-film distance. (c) Trout spinal cord, stretched by about 80% in moist air. Diagram at 4 cm specimen-to-film distance. (d) Trout sperm flagella, prepared from salt suspension, washed with ether and lightly stretched in moist air. Diagram at 4 cm. (e) Trout sperm flagella. Film heated to 50°C for 5 min before mounting. Unstretched. Diagram at 4 cm. (f) 1% acetic acid extract of perch sperm flagella. Unstretched films. Diagram at 4 cm.

1956). The pattern consistently obtained from flagella from a number of sources is of protein without any indication so far of the muscle type configuration (Pautard, 1958b).

Attempts to orient films of algal flagella by stretching have met with little success, probably because of the extensive disruption of both cells and flagella during preparation. In the case of fish sperm flagella, however, well-oriented x-ray diffraction patterns have been obtained from perch sperm flagella and particularly from more recent preparations of trout sperm flagella where buffered salt solutions have been used and the films have been carefully stretched and examined in moist air. The diffraction pattern (Fig. 4a) is characterized by a number of low-angle equatorial and near reflections (Fig. 4b and Table I) and a diffuse ring at 4–5 Å with denser areas near the meridian. These spacings are strongly reminiscent of phospholipids, and the diffraction pattern from trout sperm flagella does, in fact, closely resemble that from trout spinal cord (shown for comparison in Fig. 4c). When the films of flagella were treated with ether or other solvents, the "phospholipid" pattern disappeared. The underlying diffraction pattern was usually of unoriented protein, but from time to time stretched films of flagella prepared in buffered salt solutions and extracted with ether after drying showed the reflection at 4.65 Å on the meridian, characteristic of the cross-β configuration, together with another familiar, sharper arc at about 4.1 Å, characteristic of lipid. A diagram of this type from trout sperm flagella is shown in Fig. 4d.

TABLE I.   Low-angle reflections from flagella, equatorial and near reflections[a]

Perch Sperm Flagella[b] (Å)	Trout Sperm Flagella[c] (Å)
9.6 MS diffuse	9.4 M diffuse
16.7 M	10.7 M
36.3 W	12.9 M
44.0 VS	16.0 S
82.0 VS	22.1 VW
	35.0 VS diffuse

[a] M, medium; W, weak; S, strong; VS, very strong; VW, very weak; MS, medium strong.

[b] Specimen-to-film distance, 6 cm.

[c] Specimen-to-film distance, 4 cm.

In spite of the regularity with which the cross-β pattern appears in the photographs of solvent-treated flagella, it is uncertain whether this can be called the "natural" state, since the flagellar protein appears to be very unstable and can be changed into the β-configuration by heating at comparatively low temperatures. This is shown, for example, in the diffraction photograph in Fig. 4e, where a sharp continuous ring at 4.65 A develops when films of flagella are heated to 50°C for 5 min. This configurational instability is further illustrated in films of flagella treated or extracted with acid. Even at concentrations of 1% acetic acid, dried films, stretched and unstretched, of both acidified flagella and acid extracts show very pronounced cross-β diffraction diagrams (Fig. 4f). This feature of transformation with acid into the cross-β configuration has been observed also in whole muscles and actomyosin (Pautard, 1959).

<center>INFRARED ABSORPTION SPECTRA</center>

Although the infrared absorption spectrum of proteins gives information of a less detailed kind than that which can be obtained by x-rays, it has a certain quantitative advantage in exploring also the noncrystalline state. In the k-m-e-f proteins (but not exclusively so) there are two absorption peaks of peculiar importance. The first, at 1625 cm^{-1}, is characteristic of the β-configuration, whether the chains are extended, as in β-keratin, feather keratin, and silk fibroin, or transversely folded, as in the egg stalk of the lacewing fly, *Chrysopa* (Parker and Rudall, 1957). A second broad peak at 1655 cm^{-1} can best be classified as "configurations other than β" since it does not discriminate between the α-configuration and intermediately folded or coiled states. Astbury (1958) has commented on the relation between the x-ray diffraction diagram and the infrared absorption spectrum of native and heat-denatured egg albumin. In the muscle proteins, pure myosin shows little or no change in the spectrum after acidification or heating (for example, 100°C for 1 min), whereas actin appears to be transformed under such conditions into the β-state (Graham, Parker, and Pautard, in preparation), suggesting that the changes in the spectrum of actomyosin are associated with the globular rather than the fibrous component.

The phenomenon of the "acid shifts" in the infrared absorption spectrum was in fact first observed in fish sperm flagella. Acetic acid

extracts, acidified salt extracts and whole flagella, already referred to and illustrated in Fig. 4f as giving well-defined cross-β diagrams, all gave infrared absorption spectra with pronounced peaks at 1625 cm^{-1}. The graphs shown in Fig. 5 compare the changes in the spectra of flagellar protein and extracts with actin and myosin after exposure to 50% acetic acid vapor. The spectrum given by myosin after acidification is about the same as that of all the proteins before treatment, and the extent of the peak at 1625 cm^{-1} from flagellar sources compares with actin after similar treatment in acid vapor. The results suggest that the bulk of the protein or proteins in the flagellum undergoes marked changes after acid treatment, and in terms of muscle proteins behaves more like actin than like myosin.

SOME COMMENTS ON THE X-RAY DIFFRACTION AND INFRARED FINDINGS

The reflection at 4.65 A on the meridian of x-ray diffraction diagrams was originally reported by Astbury *et al.* (1935) in heat-denatured egg albumin, but it was not until later that supercontraction was considered in terms of transformation from the α-configuration to the "jumping cracker" cross-β form (see again Astbury *et al.*, 1959, for details). In other words, the crystallographic evidence for configurational changes of the cross-β kind has been described for *two* kinds (or
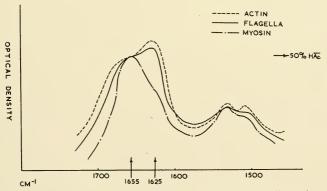
FIG. 5.   Changes in the infrared absorption spectra of flagella, actin and myosin after treatment in acid.

states) of proteins although only the α-cross-β transformation has been proposed as a basis for the change in length of the k-m-e-f proteins during supercontraction. The evidence for this change is based on the disappearance of the α-arc and the appearance of the cross-β arc in the diffraction diagrams (Rudall, 1946, 1952). The infrared evidence, on the other hand, emphasizes the crystallographic evidence that, as far as the appearance of the cross-β form in "mixed" systems is concerned, a contribution is likely to be made by *globular* proteins. Indeed, in muscle and bacterial flagella, the cross-β state which is found naturally or produced artificially is just as likely to have been contributed by the actin-like portion as by the myosin-like portion. On the other hand, the disappearance of the α-photograph in the diffraction diagram must be accounted for if any alternative explanation for the molecular changes during supercontraction is to be put forward. Some experiments on the α-keratoses extracted from oxidized wool and hair tend to suggest that for some reason, stable α-proteins can be quantitatively recovered from mixed systems where only the β or cross-β configuration can be detected in the x-ray diffraction diagram (Pautard, unpublished results; see also Pautard and Speakman, 1960). In muscle proteins, where the actin component more readily gives the cross-β diffraction pattern, the actual change in length has been associated with the fibrous myosin component. The evidence for changes in length of *actin* will be referred to later.

PREPARATION AND PROPERTIES OF CONTRACTILE PROTEINS FROM FLAGELLA

Engelhardt (1946) designated sperm ATPase as "spermosin" and suggested that the contractile protein in the flagellum might be some form of myosin. More direct evidence of the role of ATP in the movement of sperm "models" has been put forward by Hoffmann-Berling (1955) and by Bishop and Hoffmann-Berling (1959), who have drawn attention to the analogies between the extracted "model" systems and the glycerized myofibril. On the other hand, there is no clear evidence of the relationship between the flagellar proteins and those from muscle. Burnasheva (1958) failed to extract actin from bull sperm tails but reported the separation of a myosin-like protein from the same source. In contrast, Child (1959) considered the flagellar protein to be actin-

like on the basis of its nucleotide binding, while Tibbs (1957) re-
ported that the ATPase activity of the flagellum does not resemble
that of myosin.

One characteristic feature of actomyosin is the capacity to undergo
intense shrinkage when ATP is added to it in the presence of suitable
concentrations of salts. The syneresis of actomyosin has received con-
siderable attention since the original experiments by Szent-Györgyi
(1942), and Weber (1957) has more recently reviewed the subject.
Myosin alone does not contract in the presence of ATP, but when
coprecipitated with actin the combined proteins shrink, but only
when, for example, KCl and divalent cations are present. This effect
(and attendant viscosity changes) has been demonstrated in acto-
myosins from various sources, from *Amoeba* (Kriszat, 1950) and iso-
lated cell cytoplasm (Hoffmann-Berling, 1954) to many types of mus-
cle. Bishop (1958a) has reported the isolation from bull testes of pro-
teins contractile to ATP.

PREPARATION OF GELS FROM FLAGELLA

Very little protein could be extracted from fresh, damp fish sperm
flagella treated with 0.3M KCl buffered to pH 6.8 with phosphates
(Guba-Straub solutions, see Guba and Straub, 1943, for origins). This
reagent might be expected to extract some actomyosin as well as
myosin from such finely divided material as flagella, since the extent
of comminution is important in deciding the nature of the extracted
proteins. (Weber and Portzehl (1952) comment on the factors in-
volved, for instance.) Even so, no appreciable precipitate was formed
when these salt solutions were diluted tenfold after several days ex-
traction. Occasionally, however, a few microscopic fibers were thrown
down on dilution, and dialysis of the diluted extract followed by
drying of the salt-free dialyzate gave residues too small to be ex-
amined. Addition of ATP to the extracting solution did not increase
the yield.

On the other hand, extraction of flagella with 0.5–0.6M KCl buff-
ered to pH 8–9 with sodium carbonate/bicarbonate (Weber-Edsall
solutions) resulted in the removal of some 25–40% of the weight of
the flagellum, depending on the length of extraction, presence of
ATP, temperature, etc. Since more protein could be extracted from

flagella that had not moved than from those that had, all later extractions were made from flagella prepared from immobilized sperm. The alkaline-salt soluble proteins could not be precipitated, however, by lowering the ionic strength. At high concentrations, a finely divided floc occasionally sedimented after cooling the diluted solution at 2°C for several days, but more usually the solution merely became opalescent. Dialysis of these alkaline-salt extracts, whether diluted or not, always resulted in a floc separating after two or three days treatment at 2°C. A small portion of these flocs could be redissolved in 0.5M KCl after 24 hr extraction and further precipitated by lowering the ionic strength. Addition of ATP and salts at pH 7.0 to the extract before lowering the ionic strength resulted in an increase in the sedimentation rate of the floc that formed. Although the x-ray diffraction pattern of these preparations showed undifferentiated protein, microscopic, birefringent crystals, soluble in ether and partly soluble in acetone and benzene, were often seen, suggesting lipids. Present also in these dialyzed precipitates were many flat irregular plates that showed no birefringence but could be etched with ether to leave a framework of insoluble material.

It was found that dense precipitates could be formed from the diluted alkaline-salt extracts of fish sperm flagella by lowering the pH to around 5.0. Even when the solution was very dilute and optically clear, fine precipitates were often thrown down in less than a minute at pH 5.0. These flocs, when centrifuged and taken up in neutral buffers, reacted with ATP and electrolytes and formed the subjects for studies of contractility. A typical preparation of gel material from trout sperm flagella is as follows.

The flagella, damp with buffered 0.03M KCl, were extracted with 0.5M KCl containing 0.01M Na$_2$CO$_3$ and 0.04M NaHCO$_3$ for 48 hr at 2°C. The extract was separated from the insoluble residue by centrifugation at about $10,000 \times g$ for 5 min, and after removal with a fine pipet was diluted tenfold with ion-free water and cooled to just above freezing point. Saturated KH$_2$PO$_4$ was then added drop by drop until at about pH 5.2 the already opalescent solution precipitated a granular floc. This was spun down lightly for a few seconds and gently suspended in 0.1M KCl buffered to pH 7.0. A few hours after preparation a number of coherent gelatinous granules, varying from 0.01 mm–1 mm in diameter were present in the sedi-

ment, and these were all useful for subsequent experiments. Similar preparations, made in the same way from low concentrations of protein from the alkaline-salt extract of fish and rabbit muscles, were used for controls.

Gel specimens made in this way from flagella varied in their reactivity, some responding to ATP and electrolytes several weeks after preparation, others showing little or no activity even when freshly made. The behavior of the gels was studied in open pools of reagents on glass slides or in sealed cells. The results were recorded as optical micrographs or on 16-mm film in cinephotomicrographic equipment at 8 or 16 frames/sec or with time lapse up to 1 frame every 15 sec.

STRUCTURE AND PROPERTIES OF GELS

The floc thrown down upon acidification of flagellar extracts appeared to possess no fine structure when viewed in the electron microscope but showed up as a spongy, loosely aggregated meshwork, illustrated in Fig. 6a. Like actomyosin, however, these precipitates showed a tendency to form filamentous networks. Small changes in pH, and to a much lesser extent decrease in concentration of electrolytes, resulted in extension of these gels into fine filaments. Sometimes, in freshly prepared flocs, filament formation was very striking and complete, the dense, opaque, and often rounded gel specimen extending into an anastomosed network of many interwoven, threadlike bridges connecting granular islands. This appearance, which is shown in the optical micrograph in Fig. 6b, was repeated at the electron microscope level, the ultrastructure again consisting apparently of thin filaments of molecular dimensions connecting denser islands of amorphous material (Fig. 6c). Such filamentous forms could be returned partially or completely to the original "globular" state by returning the pH to about 6 or by increasing the electrolyte concentration. The ability to form filaments diminished as both flagellar and actomyosin precipitates aged until the specimens became inert. That the extended filamentous form might be due to actual molecular rearrangement into fibers seems to be supported by the frequently observed fact that bundles of filaments were often highly birefringent, the birefringence disappearing as the filaments retracted and reappearing as the filaments were extended.
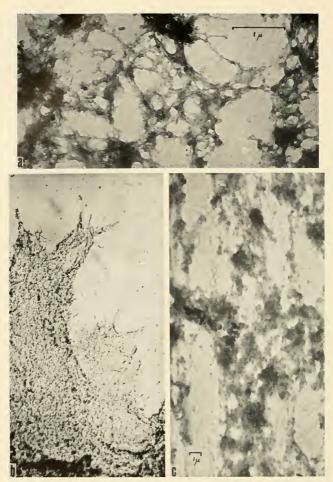
Fig. 6. Structure of flagellar gels. (a) Electron micrograph of neutralized flock from acidified alkaline-salt extract of trout sperm flagella. Unshadowed (×17,400). (b) Optical micrograph of network produced from a gel specimen from trout sperm flagella at pH 4.0 in the absence of salts. Stained in gentian violet (×300). (c) Electron micrograph of specimen (b). Unshadowed (×34,400). [*Note added in proof.* Scale in (c) should read 0.1 μ.]

206

The general effect of pH and electrolytes upon the precipitates seemed to depend on some disturbance of the internal molecular relationships, with the result that the gel specimens became unstable and filamentous under a wide variety of conditions. In contrast to these findings, no filament formation has been observed in flocs of pure myosin.

When neutral ATP alone was added to flagellar gels, they became filamentous in much the same way as they did with pH and electrolyte changes. In very fresh material washed free of electrolytes, filament formation was often very spectacular, the gel suddenly extending and streaming into long, thin webs, sometimes completely dispersing in the liquid. A "streaming" network of this kind produced at a higher concentration of ATP is shown in Fig. 7. In this particular specimen, the attenuation of the gel was so great that it was a quarter of an hour before the network was stable enough to permit photography with the rather slow plates that were used. After storage for a day, this same preparation no longer became filamentous on addition of ATP.

In the presence of KCl, however, both actomyosin and flagellar precipitates contracted when ATP (with added MgCl₂) was added. A typical syneresis of actomyosin under these conditions is shown in

FIG. 7.   Attenuated filamentous network from a gel from perch sperm flagella treated with 0.012M ATP.
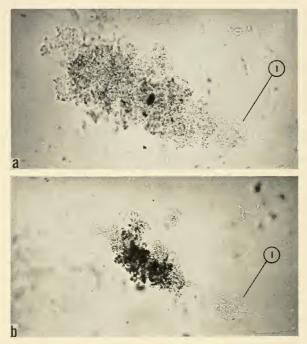
FIG. 8. Contraction of perch actomyosin with ATP. (a) Gel specimen of perch actomyosin, prepared by acidification of dilute alkaline-salt extract of perch dorsal muscle. In buffered 0.1M KCl solution (×50). (b) Gel specimen shown at (a) 2 min after addition of ATP (0.003M approximately) and MgCl₂ (0.002M) in 0.1M KCl solution, pH 7.0. Some parts of the gel [encircled 1 in (a) and (b)] have remained inert (×50).

Fig. 8. The original gel, in buffered 0.1M KCl, shown in Fig. 8a shrank in 2 min to the contracted form shown in Fig. 8b. An important feature of actomyosin-gel shrinkage that must be emphasized is that syneresis does not take place either with uniform speed or with uniform dimensional changes throughout the gel. Very frequently, parts of the specimen do not contract at all (the portion of the gel encircled 1 in Figs. 8a and 8b, for instance). This unequal shrinkage

is particularly true of flagellar gels, where the most commonly observed feature after addition of ATP, etc., in the presence of 0.1–0.2M KCl is a change of *shape*. This change in shape is shown in Fig. 9*b* in a flagellar gel prepared and activated in the same way as the actomyosin gel illustrated in Fig. 8. Here again some parts of the specimen have contracted markedly while others have remained unchanged.

Fig. 9.   Contraction of flagellar protein with ATP. (*a*) Gel specimen from perch sperm flagella, prepared by acidification of alkaline-salt extract in the same way as the perch actomyosin shown in Fig. 8. In buffered 0.1M KCl solution ($\times 100$). (*b*) Gel specimen shown at (*a*) 10 min after addition of ATP (0.003M approximately) and MgCl$_2$ (0.002M) in 0.1M KCl solution, pH 7.0 ($\times 100$).
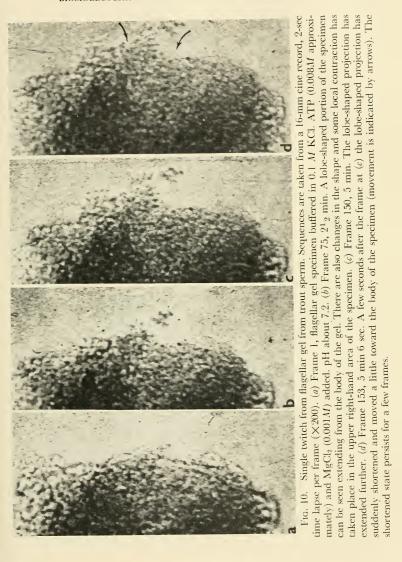
OSCILLATION OF GELS
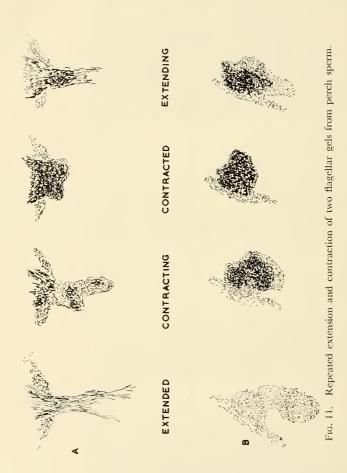
A phenomenon that appeared to be peculiar to flagellar gel specimens was the sudden commencement of rhythmic oscillation of a portion of the specimen in certain circumstances. This activity was very sporadic and seemed to depend on critical conditions for success. In general, the concentrations of ATP were higher (0.008M) than those normally used (0.003M), the pH was above neutrality (7.4 or thereabouts), and the concentration of KCl was less than usual (about 0.05M). In these circumstances, the gel specimen changed shape as usual, but sometimes part of the gel envelope extended and contracted in a regular manner, a slow extension followed by a sharp shortening twitch and a further extension, and so on. A typical "twitch" of this kind of a gel from trout sperm flagella is shown from a 16-mm cine record in Fig. 10. In the frames at 10a, 10b, and 10c, the extension of a portion of the gel envelope over 5 min can be seen. The frame at 10d shows the sudden change in position of the gel extension (indicated by the arrowing) 6 sec (3 frames) later. The complete event covers about 10 sec (5 frames), but the speeding-up of the time lapse exposure during projection makes the event appear as a sudden twitch of the gel. This kind of activity has been observed for one or more areas of a specimen, but it occurs at irregular intervals only and in occasional specimens from each preparation.

Sometimes, however, the oscillatory behavior is more regular, and two observations are drawn in the series in Fig. 11A and B. In the first event shown in Fig. 11A, a filamentous projection of the gel envelope showed 16 cycles of extension and retraction with a period of 5 sec, while in another case (the longest recorded) no fewer than 51 cycles of 3 sec period were observed in a lobe-shaped projection from a fresh gel preparation (Fig. 11B).

CONTRACTILITY OF GELS PRECIPITATED WITH MYOSIN

"Synthetic" actomyosin can be made from actin and myosin prepared separately and mixed in various proportions before precipitation. Not only can actin and myosin from the same animal be made to combine, but actins and myosins from different animals can be coprecipitated to form gels which shrink in KCl solutions when ATP

FIG. 10.   Single twitch from flagellar gel from trout sperm. Sequences are taken from a 16-mm cine record, 2-sec time lapse per frame ($\times 200$). (a) Frame 1, flagellar gel specimen buffered in 0.1 M KCl. ATP (0.008M approximately) and MgCl$_2$ (0.001M) added, pH about 7.2. (b) Frame 75, $2\frac{1}{2}$ min. A lobe-shaped portion of the specimen can be seen extending from the body of the gel. There are also changes in the shape and some local contraction has taken place in the upper right-hand area of the specimen. (c) Frame 150, 5 min. The lobe-shaped projection has extended further. (d) Frame 153, 5 min 6 sec. A few seconds after the frame at (c) the lobe-shaped projection has suddenly shortened and moved a little toward the body of the specimen (movement is indicated by arrows). The shortened state persists for a few frames.

EXTENDING

CONTRACTED

CONTRACTING

EXTENDED

A

B

Fig. 11. Repeated extension and contraction of two flagellar gels from perch sperm.

is added. In order to find out if coprecipitates could be formed from alkaline-salt solutions of flagella and muscle protein, extracts from trout sperm flagella were added to trout and rabbit actin (kindly prepared by Dr. G. N. Graham by the methods of Bárány et al., 1957) and the ionic strength of the mixture lowered to one-tenth the original value. Similarly, flagella extracts were added to trout and rabbit myosin (made according to the recommendations of Kessler and Spicer, 1952), and the ionic strength was lowered as before. Myosin, actin, and flagella solutions alone were diluted as controls, and myosins and actins were coprecipitated and used to test the activity of the combination.

The results suggest that the myosin/flagella precipitates were more reactive to ATP than myosin or flagellar protein alone, and the supernatant after precipitation of the myosin/flagella mixture gave no precipitate when acidified to pH 5.0. On the other hand, no precipitate was thrown down by the actin (actin normally lowers the solubility of myosin in salt solutions), and the precipitate from this solution after acidification to pH 5.0 was no more active than the flagellar precipitate alone. Although this does suggest that some combination might have taken place between myosin and flagellar protein to give a synthetic actomyosin of sorts, it is equally possible that coprecipitation with myosin has simply improved the mechanical arrangement of the flagellar gel and the myosin has a passive role.

OTHER EXTRACTION RESULTS

Failure to extract protein with neutral $0.3M$ KCl suggests that little myosin might be present. At the same time, no protein (or at best, only traces of protein) have been extracted from whole flagella and flagella residues by the methods of Bárány et al. (1957) for the preparation of actin.

However, whereas extraction experiments with unicellular algae showed that the cell cytoplasm as well as the flagella contained contractile protein, extraction of fish sperm heads resulted in the removal of very little (less than 0.1%) material, too small to be examined and certainly within the limits of experimental error.

After extraction of fish sperm flagella with alkaline salt solutions, examination of the residue in the electron microscope showed that the subfibrils, although disordered and fragmented, seemed to be still

present. Even after several days extraction, the 9 plus 2 arrangement could often be detected, and although no information was obtained as to the site from which the contractile protein had been removed, there was also no evidence that obvious structures in the flagellum were involved.

GENERAL CONCLUSIONS

The results of experiments on fish sperm tails suggest that a protein which reacts with ATP like a very feeble actomyosin can be extracted from the 9 plus 2 flagella but not from the heads. This protein does not behave characteristically like a myosin since myosin extractants remove no material from the flagellum and there is no x-ray diffraction evidence of the meridional α-reflection at 5.1 A. The changes in the infrared spectrum after acidification suggest that the whole flagellum and its extracts behave more like actin than myosin (and the lack of orientation in the x-ray diffraction pattern would seem to support this), but no protein can be removed from the flagellum by methods normally used to prepare actin. Although lack of knowledge of special circumstances might have prevented separation of familiar proteins from flagella, it might be that the flagellar protein is not, in fact, an actomyosin but some equally effective complex, possibly in association with lipid. Failure to extract from the sperm heads protein that reacts with ATP seems to be direct evidence that the contractile system is confined to the tail. Moreover, the oscillatory behavior of flagellar gels, apart from the implication that a "flagellum" can be reconstituted as a "model," seems to point to some fundamental mechanism in which the extension and retraction of a filamentous arrangement of molecules may be the property of a complete protein complex.

SYNERESIS OF ACTIN

Syneresis of muscle protein is generally regarded as a property of actomyosin and attempts to produce a model contractile system with myosin alone have met with little success. Not only is myosin mechanically inert to the ATP which it splits, but it also does not seem to react to electrolytes, pH changes, etc., in the way that actomyosin does. Actin "does something" to the myosin (see, for instance, the

conclusions of Hayashi *et al.*, 1958, on the role of actin in actomyosin contraction), but it is difficult to study the mechanical properties of actin alone in the way that myosin can be studied since actin is soluble and does not form precipitates or threads except under unfavorable conditions (e.g., in acetone/water mixtures). Experiments with acetone precipitates lightly fixed with formaldehyde suggested that although such crosslinked actin gels were not reactive in the sense that actomyosin is reactive, the insoluble specimens were nevertheless capable of swelling and deswelling to a much greater degree than myosin.

During an examination of films of F-actin dried down from solutions containing traces of salts, it was found that although most of these films were insoluble, the outer rims of each plaque were transparent, fibrous in appearance, birefringent, and generally oriented tangentially. Fragments of these outer portions swelled in ion-free water and with ATP. Lowering the pH to 4.0 resulted in further swelling and eventual solution. Addition of neutral KCl produced much the same effect. Diminution of the pH in the presence of KCl, however, resulted in a spectacular syneresis of the specimen, which shrank rapidly, becoming a dense gel rather like syneresed actomyosin. Three preparations of this kind before and after addition of KCl at pH 3–4 are shown in Fig. 12. In the first example (Fig. 12a and b) the actin film has shrunk after 1 min, but as in the case of actomyosin and flagellar protein treated with ATP, etc. (see Figs. 8b and 9b for comparison), some parts of the gel have remained inert. In the second example (Fig. 12c and d), intense syneresis has taken place in an actin film in 3 min, and in contracting to the dense pellet shown in Fig. 12d, a debris of fine particles has been ejected from the body of the specimen. In the third example (Fig. 12e and f) the gel specimen of actin has contracted vigorously, but once again some parts of the gel appear to be unreactive. In those birefringent films that suggested a certain amount of orientation, the swollen gels shrank along the axis of orientation, often with a folding or buckling of the film. In some instances the gels contracted to about 30% of their original length. On the other hand, specimens prepared from the center of the dried plaques or from old actin specimens were inert, and the behavior of the more reactive preparations suggests that these particular gels may represent a form of F-actin with suf-
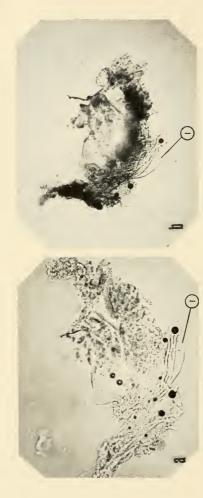
FIG. 12. Syneresis of actin gels (×72). (a) F-actin film in ion-free water. (b) Specimen shown at (a) 1 min after addition of KCl (approximately 0.03M) at pH 3.4. The parts of the gel in (a) and (b) indicated at 1 have remained relatively inert.
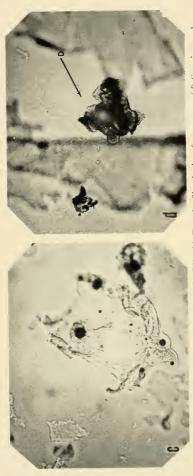
Fig. 12. (Continued.) Syneresis of actin gels (×72). (c) F-actin film in ion-free water. (d) Specimen shown at (c) 3 min after addition of KCl at pH 3.4. The gel has contracted to a dense pellet and the surrounding liquid is clouded with a debris of fine particles (arrowed D).
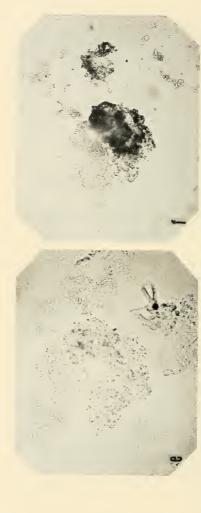
Fig. 12. (Continued.) Syneresis of actin gels (×72). (e) F-actin gel in ion-free water. (f) Specimen shown at (e) 1 min after addition of KCl at pH 3.4. As in the case of syneresed actomyosin and flagellar protein, the gel has not contracted equally in all directions.

ficient cohesion between the filaments to prevent the otherwise soluble protein from drifting apart during swelling. In this way the individual behavior of the F-actin filaments was perhaps coordinated to produce a network of reactive linear elements.

In considering the nature of contractility in living things, it is very difficult to decide which factors are merely the embellishments of evolution. In mammalian skeletal muscle there are so many biochemical and physiological features involved that it is scarcely surprising that there are almost as many theories as facts. Even in simpler organisms, the electron microscope is now revealing such fine detail in the structure of contractile organelles that it seems likely, from a molecular standpoint at least, that we may never find a "primitive" living contractile system, particularly since locomotion is a major factor in the struggle for existence and ancient forms of movement may have been swallowed up long ago by their more efficient descendants. In this scheme of things, however, the flagellum seems to have remained, by tacit phylogenetic agreement, as a blueprint satisfactory enough to stand the test of time. There is no reason to suppose that unicellular animals swam in primeval seas with any different 9 plus 2 flagella than they do today, and the modern flagellum, however modified, might still contain those common denominators once used to improve motility and to develop more advanced muscles.

Models of Contraction

Very broadly, two models of contraction have been proposed as a basis of biological movement—those based on the relative movements of rigid elements and those based on the folding or bending of some, or all, of the contractile substances. These models depend on different data, some morphological, some physical and physicochemical, and some on thermodynamic argument. There is sufficient evidence, however, to indicate that most of the living contractile systems that have been studied have a common link somewhere, and if this is so, any really fundamental explanation of contraction must apply equally to a wide range of motile organs and organelles; and moreover, the explanation should also fit contractile systems that have been reconstituted from extracted components, often from different animals.

In muscle, the "sliding" mechanism proposed by Hanson and Huxley (1955) has been developed from morphological considerations of the more advanced myofibrils, and the idea of interposition of rigid elements does receive some support from the x-ray diffraction evidence, which does not suggest any change in the molecular arrangement after shortening (Astbury, 1947a, for example). On the other hand, there are numerous models for a folding mechanism, and the subject has a long history. Although Meyer and Mark (1930) claimed to be the first to propose a folding of molecules as a primary event in muscular contraction in their objection (see Meyer and Mark, 1951) to the conclusions of Goldacre and Lorch (1950), the physical reality of a folding protein was first demonstrated by Astbury (Astbury and Street, 1932; Astbury and Woods, 1933), who showed that proteins could undergo reversible changes of dimension as a result of actual changes in the configuration of molecules. Numerous models have been proposed since, based on supercontraction (Astbury, 1960), crystalline-amorphous changes (for example Flory, 1956), Donnan osmotic and electrostatic effects (developed by W. Kuhn and reviewed by Kuhn et al., 1960).

In other biological contractile systems, the structures are so diverse that comparison with muscle is difficult. The concept of rigid elements moving over one another has been developed by Noland (1957) to explain protoplasmic streaming, while a mechanism of parallel displacement forces has been put forward by Jahn and Rinaldi (1959) to account for the movement of filaments in *Allogromia laticollaris*. In contrast, a folding model has been proposed by Goldacre and Lorch (1950) as the basis of amoeboid movement; also a number of composite sliding-folding systems have been suggested (for example, Frey-Wyssling, 1948).

Perhaps the most surprising feature of this wealth of theories on movement throughout nature is that many of them have been developed independently of any knowledge of the nature of the contractile substance, and where the substance has been identified as actomyosin, most of the theories are detailed without any reference to the properties of actin or myosin. In spite of the considerable literature on the physiology and kinetics of the myofibril, there is little information about the mechanical and mechanochemical properties of actomyosin, very little about myosin, and none at all about actin. It is almost as if the contractile system were a poor model for itself.

Relationships between Flagella and Muscle

The evidence presented above suggests that, although fish sperm flagella contain a proportion of protein that shrinks like a feeble acto-myosin with ATP, there is nothing to indicate the presence of true actin or myosin. The contractile protein differs from actomyosin, too, in the way in which it must be prepared, and in the added property of oscillation in certain circumstances. Although in the bacterial flagella there are striking x-ray relationships with skeletal muscle (Astbury *et al.*, 1955), no such similarity has yet been found in the 9 plus 2 flagella, where there is also neither morphological nor biochemical evidence for any very close comparison with the myo-fibril. The resemblances between the 9 plus 2 flagella and muscle might best be seen in the behavior of some common macromolecular arrangement, particularly in its response to ATP or equivalent electrolytes, and it is here that the soundest basis for movement might be found.

Flagellar Motion

The oscillatory activity of flagellar gels treated with ATP suggests that the property of regular contraction and relaxation may be built somehow into one system, perhaps one molecule, by the circumstances of its response to nucleotides. This effect is not unlike the oscillatory behavior of the extracted myofibril induced by creatine phosphate (Goodall, 1956) or by addition of phosphoenolpyruvate after maximal tension (Lorand and Moos, 1956). The "automatic" behavior of gels might answer Bishop's (1958b) second question: "How can a co-ordinated wave-like motion occur in cells whose permeability, ionic balance, and metabolic integrity have been destroyed?"; for although the organism may have lost most of its biochemistry and all of its supply of energy, the individual "contractile" molecules may still possess *their* permeability, ionic balance, and metabolic integrity, preserved long after other cellular processes have been disorganized. We might imagine the "contractile" molecule as a complete engine in itself, self-regulatory and tireless. In a physiological situation, ATP appears to have the dual function of relaxation at low electrolyte and high pH and nucleotide concentration, but contraction at higher electrolyte and lower pH and nucleotide concentration. In the flagellum and in the reconstituted gel

specimens, the rhythmic contraction and relaxation might be the result of those critical conditions necessary to keep the "contractile" protein moving between the two extremes. The electrolyte concentration is important for this effect and divalent ions may be essential in determining how the system will behave (see, for example, Hoffmann-Berling, 1958). Oscillation seems to depend on conditions tending to produce relaxation, so that the local environment is important. In the intact flagellum, this is best illustrated by the experiments with enfeebled sperms, where the first condition essential to a revival of activity is a swelling or dispersion of the milt, reflecting some analogous change in the cells.

The contraction of flagellar gels at low ATP concentrations in the presence of KCl and the extension of normal and contracted gels at higher ATP concentrations in the absence of KCl, coupled with the effects of pH in extending and contracting the gels from flagella and muscle, suggest that these factors might be responsible for oscillatory activity by cancellation of opposing halves of the cycle. By adjusting the concentration of ATP (or possibly of electrolyte), for instance, a local environment relaxing the system in circumstances where a local enzyme will also contract the system, may be created. In this case the system is a network of filaments decided by the geometry of the participating proteins (and possibly lipids). This reactive network is marshalled and extended, perhaps, by the overall nucleotide/electrolyte concentrations at increasing pH, but since this raises the rate of nucleotide splitting (Tibbs, 1959, finds an optimum pH of 8.2 for Mg^{++} activation in whole perch sperm flagella), the concentration of ATP and the attendant pH fall *at the site of splitting* and cause the network to contract. The loss of substrate and unfavorable local pH will then tend to depress or halt enzyme activity so that the local environment can once again take over and cause the network to reextend to start the next cycle. During the contraction phase, the network may behave as a *pump*, possibly even as a molecular pump, altering the local hydraulic conditions, ejecting reaction products, and changing the surrounding concentrations of salts, etc. A tentative arrangement along these lines is illustrated in Fig. 13.

In this scheme, it is not necessary to postulate specialized relaxing mechanisms to reverse the contraction phase, as is the case in muscle. Instead, as long as the system allows free entry and exit of participat-
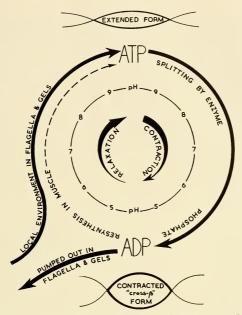
Fig. 13.   Reversible contraction of flagellar protein.

ing reagents and the necessary molecular architecture is intact, both flagellar gels and extracted sperm models will work automatically. Since one of the problems of flagellar motion is the transport of substances through the attenuated 9 plus 2 arrangement, it may be that even here there is little need for a semipermeable membrane; the *permeability of the flagellar surface may be a function of the "contractility" of the proteins.* Providing that the enzymes (or the substrate-splitting moieties) are retained strategically and the contractile arrangement is related and continuous, the flagellum will continue to beat, and the reacting substances will be transported hydraulically, just so long as the substrate is available and the requisite ions are in the vicinity. Overall control of the motion of the flagellum might be exercised by "pinching off" the supply of salts or substrate in a strategic way.

Although experiments on flagellar protein and actomyosin (Pautard, 1959) suggest that biological contraction may be due to a proton transfer mechanism (proposed also by Goodall, 1958), there are conflicting views on changes of pH in muscle during contraction. Some measurements suggest very small shifts of pH (Caldwell, 1958); others suggest appreciable values (Distèche, 1960). Since the term "pH" is meaningless when applied to a macromolecule with as many polar groups as a protein, it may be advantageous to think in terms of transfer of charges across polypeptide chains, in which case the flagellum may derive energy for movement without the actual transport of electrolytes at all. This is particularly true of bacterial flagella, where no enzyme activity or substrate has been detected (Weibull, 1948; Barlow and Blum, 1952) and there is no alternative but to consider contraction in terms of physics alone.

A Possible Universal Molecular Basis for Movement

The flagella that have been studied appear to be largely composed of unstable protein or proteins, possibly of a globular type. It may be that these proteins are unconnected with contraction, but additional physical evidence, from studies of both flagella and muscle, suggests that perhaps the globular proteins, or at least certain types of globular proteins, may be the prime movers in biological contraction. The evidence in support of this is as follows.

Whatever the morphological arrangements in muscles, flagella, and reconstituted gels, there is no doubt that the proteins involved are members of the k-m-e-f group and satisfy Astbury's (1947a) concept of contraction as part of a much wider plan throughout nature. The contractile event, one way or another, is vested in these proteins, and we need look no farther. In muscle, the problem is to decide whether *both* actin and myosin are essential for contraction in the molecular sense. In the past, a great deal of emphasis has been placed on the myosin partner, partly because of the way in which "myosin" preceded actin in the literature and partly because intramolecular configurational changes have been actually demonstrated with the α-proteins. Thus, the appearance of the strong cross-β photograph in the diffraction diagrams of heated muscle has been considered as a transformation of the myosin during supercontraction, while experiments demonstrating a "melting" of the crystalline phases during physio-

logical contraction of the myofibril similarly tend to support the idea of changes in the state of the myosin (Mandelkern *et al.*, 1959). On the other hand, the appearance of a very weak cross-β configuration in actomyosin following addition of ATP (Pautard, 1958b) or with physiological changes of pH (Pautard, 1959) has recently been attributed to changes in the actin rather than the myosin. Alternative evidence of the stability of the α-configuration in certain circumstances comes from experiments with α-keratoses extracted from oxidized wool and hair. Whereas these α-proteins show neither mechanical nor configurational changes with changes in pH, the residual β-keratoses (which have no apparent structure when extended) are highly contractile and show configurational changes under the same conditions (Pautard and Speakman, 1960).

These experiments suggest that the globular proteins are more readily capable of configurational change than the fibrous α-proteins, and it is tempting to suggest that when α-proteins do supercontract *and* give a cross-β diffraction diagram they must first go through a globular phase, so that the common progenitor, so to speak, is some intermediate state where the protein is neither one thing nor the other. This may be the special significance of proteins like actin, where globular units are aggregated into fibrous polymers. Experiments with F-actin gels which show that actin *by itself* is capable of considerable dimensional change at pH values producing the cross-β configuration suggest that what happens to α-proteins under vigorous conditions is precisely what happens to F-actin under physiological conditions; "supercontraction" is simply applicable, *in extenso*, from one to the other. In other words, since every k-m-e-f protein may be both fibrous and globular (see Astbury, 1958, for correlation), F-actin will undergo the same configurational and dimensional changes as those observed during the physiological syneresis of actomyosin, whereas myosin will do so only after more drastic treatment.

In considering how the actin may undergo dimensional change, it must be noted that whereas it is customary to illustrate a macromolecule as a thin line, without reference to its shape, globular proteins, which are mixtures of regular and random regions coiled together in various ways, are best illustrated at several levels of structure—the straggling polypeptide chains, the more ordered helical portions and the outline of the whole molecule. In G-actin, the molecule is an

ellipsoid with a low (8%) helix value (Kay, 1960) possibly due to the high proline content (Kominz *et al.*, 1954), and although there is disagreement about the state of aggregation of the G-form (Kay, 1960), the conditions of polymerization into the F-form have been well established. Contraction of a string of molecular beads of this kind will depend on the behavior of the polypeptide chains coiled within each bead with respect to the fiber axis, and if the network formed with this kind of fiber has weak side-to-side linkages, then the contraction of the network is the sum of all the filaments sliding over each other. If the side-to-side linkages are too weak, the protein will go into solution. If the filaments are strongly crosslinked, the network will be constrained and inert. Syneresis may presuppose *only* linear linkages, and in muscle and flagella, this may be the special property of the globular component—where typical globular behavior is accurately synchronized by the arrangement of the end-to-end linkages. In serum albumin, Bresler (1958) found an increase in the axial ratio from 4 to 16 at pH 10 when the solvent was made more hydrophobic, and similar intraglobular transformations in a linear polymer would lead to marked changes in length (Astbury, 1958).

Bresler (1958) also found no changes in optical rotation during changes in shape of serum albumin. This suggests that the helices took no part in the process. Changes of pH apparently have little effect on helices too, so that in actin, the more random and freely movable portions of the molecule may be responsible for the changes in length, leaving the more stable helical regions to move among themselves. There is no indication of the way in which the cross-β configuration is generated by this system, and further experiments are needed before speculation on the molecular arrangement is possible. It seems clear, however, that these kinds of globular molecules have the dual function of a supporting skeleton with movable arms and legs.

Actin does seem to conform to the kind of structure that has been postulated by many authors as an electrostatic and osmotic engine, but there is no evidence to suggest how the principles that have been derived from considerations of polyelectrolytes (Katchalsky, 1951, for instance) fit into the behavior of the actin or actomyosin networks. Suffice to say, the changes in actin (and flagellar gels) during contraction and extension have some features apparently unconnected with

structural changes, and others (like the cross-β state when the molecule is completely contracted) that are characteristic of the configurational changes of the k-m-e-f group of proteins.

The Position of the Flagellum in the Evolution of Movement

The proteins present in the flagellum are distributed in some unknown way so that their effect in producing wave motion cannot be deduced. There is no evidence in the 9 plus 2 system for a sliding mechanism of rigid elements (Gibbons and Grimstone, 1960); there may not be any continuity of contractile protein at all, but rather attachment of separate molecules in a geometric manner to some insoluble basement. In this respect, it must be pointed out that since the flagellar protein may be highly solvated, shrinkage after fixation and drying for examination in the electron microscope might tend to create fiber-like artifacts from diffuse and swollen gels. This may be true in muscles, and in flagella, certainly, considerable disagreement has arisen already on the subject of the 9 thinner filaments reported by Gibbons and Grimstone (1960) and others (Afzelius, 1959; Fawcett, p. 149). In one sense, the flagellum may represent more than a simplified muscle. It may embody arrangements used earlier in the history of movement, since there may have been an evolution of actin-like molecules from simpler forms. Members of this molecular family may have similar properties. They can be polymerized end-to-end and depolymerized to form an endless variety of structures. They can form filaments alone, or they can be associated with more stable fibrous structures (perhaps even in the complex mammalian sperm) acquiring direction and purpose, particularly if enzymes or substrate-splitting sites are located at specific points in the fibrous (e.g., myosin) partner. In muscle, the actin may be in the F-form permanently (Martinosi, Gouvea and Gergely, 1960); but elsewhere, the end-to-end association might be temporary, allowing endless fabrications of pseudopodia, reticulopodia, and other organelles as required.

Originally, perhaps, these molecules may have been of the "portable" kind, but as they became more specialized for a particular type of motion, the attendant biochemistry doubtless became more and more complex and the reactive proteins were fixed relative to one another. The flagellum may have been an early example of specialization directed toward rapid and controlled movement—the first

"muscle." To do this, the random systems had to be ordered and made stable. But what of these random systems? Perhaps these were the result of a much longer evolution of unstable "globular" molecules, which in themselves possess a kind of molecular predation. Indeed, the flagellum itself might be an early attempt at peaceful coexistence after a long period of bitter struggle between rival contestants.

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Adenosine Triphosphatase and Acetylcholinesterase in Relation to Sperm Motility

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Much time and thought has been devoted to studies on striated muscle cells. Although there is no general agreement as to the mechanism of action of these cells, certain features must certainly have a place in this mechanism. Nature may well have used some of these ideas in her design for sperm motility, and thus one approach to this problem is to search for evidence of such common features.

Structurally, striated muscle has been revealed in considerable detail by the work of Huxley (1957), who has also taken a part in presenting us with stimulating information regarding the localization of particular proteins (Huxley and Hanson, 1957). The fine structure of the sperm tail has been discussed authoritatively during this symposium. Comparisons of biophysical features in muscle and sperm tails do not, in fact, seem to be particularly fruitful. This is hardly surprising since even a common biochemical mechanism, were such to exist, would have to be arranged very differently in each case to produce the two vastly different types of motion shown by the muscle cell and the sperm tail. This paper will be concerned with some of those biochemical features which are the ones most likely to be included in any common basic mechanism.

ADENOSINE TRIPHOSPHATASE ACTIVITY OF THE SPERM TAIL

During the movement of a muscle cell two phases of activity may readily be distinguished. The first is an active phase in which the cell and the component myofibrils contract. At the same time enzymic hydrolysis of adenosine triphosphate (ATP) takes place. The second

phase is a more passive one of relaxation in which the cell regains its original form. This relaxation phase is also characterized by a low rate of ATP splitting. In the case of the sperm tail during motility no such ready division into, or observation of, two such phases of activity is possible. If such phases exist, some components must be contracting and others relaxing at the same time. Moreover, a particular point on the tail must be involved in a continuous cycle of contraction and relaxation.

A high rate of ATP hydrolysis is characteristic of the contraction process in muscle but Marsh (1952) pointed out that, in contrast with this, relaxation is accompanied by a low rate of ATP splitting. Indeed, it seems that enzyme inhibition and a resulting low rate of hydrolysis is a prerequisite for relaxation. High concentrations of ATP relax muscle and have been shown to inhibit the enzyme when these concentrations exceed those of the magnesium activator (Perry and Grey, 1956). *In vivo* the inhibition is brought about through the agency of a factor discovered by Marsh (1952) and widely known as the "Marsh factor" or "relaxing factor." The mode of action of the factor is uncertain but it seems likely that it acts by chelating some essential metal activator (Baird and Perry, 1960), whereas inhibition by excess substrate may be explained by the substrate playing a similar role.

Sperm tails and protozoan flagella which have a similar morphology possess magnesium-activated adenosine triphosphatase (ATPase) activity (Nelson, 1954; Tibbs, 1957); estimates of the amount of energy needed to propel the cell through water show that the measured rates of ATP breakdown by flagella *in vitro* are quite high enough to provide the energy needed for propulsion (Tibbs, 1957; Nelson, 1958). It is then relevant to ask if the movements of the flagellum are controlled and governed by the activation and inhibition of the ATPase. Or, to put the question in terms which can be investigated experimentally, will excess ATP cause enzyme inhibition and is there any evidence to suggest the presence of a factor in sperm which may inhibit the ATPase of the tail?

Some information is available on these questions. When the influence of substrate to activator ratio was investigated on sperm tails obtained from the common perch (*Perca fluviatilis*) results such as those shown in Fig. 1 were obtained (Tibbs, 1959). It is apparent that although enzyme activity decreases with increasing substrate concen-

tration after a maximum value has been obtained, the decrease is far less dramatic than in the corresponding case of the myofibril, and it is hardly possible to believe that this inhibition is basic to an effective relaxation mechanism. It would be of interest to consider the effect of muscle relaxing factor on the sperm tail ATPase, but results on this aspect of the work have not, up to date, been of much use since the muscle relaxing factor is unfortunately associated with a granular fraction (Portzehl, 1957) which itself carries an ATPase. In estimations where the myofibrillar enzyme is mixed with relaxing factor, the ATPase of the latter is a good deal smaller and can be allowed for. The sperm tail enzyme is much less active, and in suspensions which contain added muscle relaxing factor the enzyme activity of the latter is dominant. Nevertheless the idea may be worth pursuing in view of the fact that Bishop and Hoffmann-Berling (1959) report that muscle relaxing factor inhibits the flagellation of sperm models.

Attempts have also been made to obtain evidence for the existence
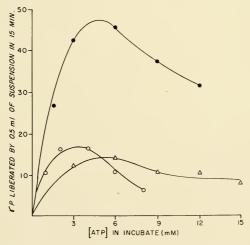
Fig. 1.   Effect of substrate concentration on the splitting of ATP by three preparations of flagella. Carried out in 0.1M borate buffer pH 8.2. ○, in presence of 2.5 mM MgCl$_2$; ●, in presence of 5.0 mM MgCl$_2$; △, in presence of 7.5 mM MgCl$_2$.

of a flagellar relaxing factor. Various fractions from the milt of the brown trout (*Salmo trutta*) were examined to find out what effect, if any, these fractions had on the flagellar ATPase. These results are summarized in Table I. Potassium oxalate (2.5 mM) was normally included in the activity determinations since calcium ions are known to inhibit the muscle relaxing factor (Bozler, 1952). Minimal concentrations of magnesium activator were employed, and therefore any inhibition arising from magnesium chelation (Perry and Grey, 1956) should have been detectable. The "final supernatant" of the third column was the supernatant obtained from the spermatozoa after these had been homogenized in $0.05M$ potassium chloride (which in the case of rabbit muscle would extract the relaxing factor) and the heads and tails removed from this suspension by centrifugation.

It appears from the results that no one fraction is capable of causing enzyme inhibition. The possibility that a relaxing factor may be firmly attached to the flagellum and may be extracted only with difficulty cannot be ignored. Indeed the high degree of control and the fact that one part of the flagellum may be relaxing while another part is contracting may be urged as indicating the probability of a firm attachment of a factor at significant points. However, as a consequence of this, in activity estimations, the sperm tail would show the reduced activity. The measured activity is always entirely adequate to account for the energy required (Tibbs, 1957; Nelson, 1958), and no anomalous results have ever been obtained to suggest that under certain circumstances the enzyme may possess enhanced activity corresponding to the accidental removal of a relaxing factor. The evidence in this case, therefore, indicates the absence of an ATPase inhibitor in trout milt and of a relaxing factor operating by a process involving enzyme inhibition.

Whatever the actual mechanism of flagellation, it seems evident that two processes must be involved. In one of these, ATP is split, and the energy liberated is used for motility. In the second process, ATP is not hydrolyzed, but those events taking place on ATP splitting are reversed, and the component in question returns to its original condition. In the case of muscle, the cell models of Hoffmann-Berling (1954), and the tail of the bacteriophage T_2 adsorbed on the host (Kozloff and Lute, 1959), the process of ATP splitting is accompanied by contraction. This is not a universal rule in systems show-

TABLE I. Effect of trout milt fractions on flagellar ATPase[a]

Preparation No.	Seminal Plasma				Cell Heads				Final Supernatant			
	Potassium oxalate (mM)	Magnesium Chloride (mM)	Concentration (vol. %)[c]	Inhibition (%)[b]	Potassium Oxalate (mM)	Magnesium Chloride (mM)	Concentration (vol. %)[c]	Inhibition (%)[b]	Potassium Oxalate (mM)	Magnesium Chloride (mM)	Concentration (vol. %)[c]	Inhibition (%)[c]
3T	2.5	0.63	8	+7								
	2.5	1.25	8	0								
	2.5	2.50	8	−4								
4T	2.5	0.63	8	−20					2.5	0.63	4	−30
	2.5	1.25	8	−3					2.5	1.25	4	−33
5T					2.5	0.63	4	+38				
					2.5	1.25	4	+26				
6T					2.5	0.63	4	+16	0.0	0.63	4	0
					0.0	1.25	4	+6	0.0	1.25	4	+12
									0.0	2.50	4	−4
									0.0	5.00	4	−2
7T	2.5	0.63	8	−14	2.5	0.63	4	−14	2.5	0.63	4	−20
	2.5	1.25	8	+2					2.5	1.25	4	−7
	2.5	2.50	8	+27					2.5	2.50	4	+5
8T	2.5	0.63	16	−16	2.5	1.25	8	+35				
	2.5	1.25	16	−22	2.5	2.50	8	+29				
	2.5	2.50	16	−4								
	2.5	5.00	16	−30								
9T									2.5[d]	1.25[d]	16[d]	+6[d]
									2.5[d]	2.50[d]	16[d]	−1[d]

[a] Measured in 0.05M tris buffer, pH 7.8 and 2 mM ATP with magnesium activator, 15 min incubation at 37°.

[b] Positive sign indicates inhibition, negative sign an activation.

[c] Concentration expressed in terms of volume of milt from which obtained.

[d] This particular fraction was an extract of the plasma-depleted milt in 0.05M KCl. There was no homogenization and the sperm were removed intact.

237

ing motility since Hoffmann-Berling (1958) has also shown that in glycerized models prepared from the stalks of *Vorticella gracilis* calcium ions but not ATP induce contraction, whereas the addition of ATP to the contracted models causes relaxation and with calcium ions still present sets up a cycle of alternate contraction and relaxation.

In the case of the sperm tail it is not easy to say whether ATP is split during a contractile process or not. The extraction and behavior of functional material should do something to clarify the situation. Pautard (1957) has described an extract from perch sperm flagella which forms a gel contracting or extending in ATP depending upon whether potassium chloride is present or not. Bishop (1958a) has obtained from bull testes a preparation which hydrolyzes ATP and undergoes superprecipitation in the presence of ATP and magnesium ions. This last experiment seems to link contraction with the ATP-splitting step.

In the case of muscle, inhibition of the ATPase by either natural or artificial systems results in relaxation, and activation of the enzyme in the presence of substrate produces contraction. It seemed likely that by inhibiting the flagellar ATPase before the sperm had been allowed to swim, it might be possible to prepare tails in which all the contractile material was in the same state, that immediately preceding ATP breakdown. Activation of the enzyme of this preparation in the presence of ATP might then produce a second state, the two states being distinguishable from each other by a change in flagellar volume. There existed then the possibility of detecting such a difference by the optical method of Cleland (1952), which has been used to measure the swelling of mitochondria.

The first requirement for this experiment was a system which could be used to inhibit the flagellar enzyme reversibly. *p*-Chloromercuribenzoate, found by Engelhardt and Burnasheva (1957) to inhibit an ATPase preparation from sperm, did not produce a high enough degree of inhibition to warrant a more detailed investigation into the reversibility of its action.

In the absence of activator, the sperm tail enzyme has a much reduced activity (Tibbs, 1959). Pyrophosphate and ethylenediaminetetraacetic acid (EDTA) both inhibit the ATPase of muscle presumably by the removal of activator, although in the case of EDTA the in-

hibition occurs at concentrations which are far too low to chelate all the magnesium in the system (Perry and Grey, 1956). The effect of pyrophosphate and EDTA on the sperm tail enzyme was determined. At concentrations of magnesium ions comparable to or less than the pyrophosphate concentration, some inhibition occurred, but EDTA was a far more effective inhibitor, and further experiments were confined to the use of this substance.

Enzyme measurements and the subsequent work on changes in flagellar volume were all carried out in pH 7.8, 0.05M tris buffer containing 0.5M sucrose. The flagella themselves were isolated in this medium from milt diluted with the same tris buffer containing both 0.5M sucrose and 2.5 mM EDTA. Although this medium seemed to permit a reasonably high degree of morphological integrity, no claim is made that it is the most suitable for the purpose.

Figure 2 shows the effect of EDTA on the perch sperm ATPase, the EDTA being added prior to the magnesium activator to indicate the
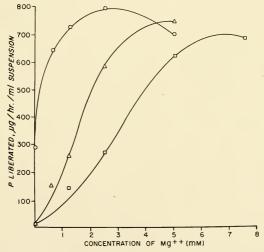
Fig. 2.   Effect of Mg^{++} activator on the sperm tail ATPase in the presence of EDTA. Incubation at 37° for 10 min in pH 7.8, 0.05M tris buffer containing 0.5M sucrose and 2 mM ATP. ○, no EDTA; △, 1.25 mM EDTA; □, 2.5 mM EDTA.

reversible nature of the effect. In 1 mM $MgCl_2$, 1.25 mM EDTA reduced the enzyme activity to about 25% of its original value and 2.5 mM EDTA lowered it still further to about 10%. On the other hand, increasing the concentration of $MgCl_2$ above that of the EDTA produced a rapid increase in activity to give approximately the same maximum value in each case. By altering the magnesium to EDTA ratio, the enzyme could therefore be activated or inhibited as required.

The use of turbidity measurements for showing changes in the water content of mitochondria has been described by Cleland (1952). If transparent particles, suspended in water, are made to absorb water, then their refractive index approaches that of water, scattering of light is reduced, and the turbidity of the suspension is lowered.

Figure 3 shows the effect on the optical density at 520 mμ of activat-
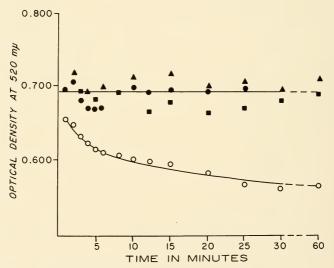
Fig. 3. Change in optical density at 520 mμ with time (room temperature throughout) after mixing perch sperm tails with $MgCl_2$ and ATP. Each cuvette contained the same quantity of flagella in standard sucrose-tris solution, and final suspensions were also 1.6 mM with respect to EDTA. ●, no $MgCl_2$, no ATP; ▲, 5 mM $MgCl_2$, no ATP; ■, no $MgCl_2$, 2 mM ATP; ○, 5 mM $MgCl_2$, 2 mM ATP.

ing the ATPase of a perch sperm tail suspension in the presence of substrate. A reduction in optical density of between 15% and 20% occurred. This seems to indicate a swelling of the sperm tails. Out of a total of 17 preparations, 10 were active to about the extent just described, 3 had small activity, and 4 were inactive. Calcium but not barium could replace magnesium as activator, the effect taking place at a much reduced rate. Calcium will also activate the flagellar ATPase but is less effective than magnesium (Tibbs, 1959). Adenosine diphosphate (ADP), adenosine monophosphate (AMP), inosine triphosphate (ITP), sodium pyrophosphate, and sodium triphosphate were all tested for their ability to replace ATP. ADP replaced ATP quite satisfactorily, the other substrates being without effect. ADP was also hydrolyzed by the sperm tail preparations about 30% as effectively as ATP. This is in line with the observations of other workers (Nelson, 1954; Engelhardt and Burnasheva, 1957), although in the past the author has had preparations which did not seem to hydrolyze ADP (Tibbs, 1959).

To provide more convincing evidence that the optical density change was really connected with ATP hydrolysis the effect of altering the $MgCl_2$ in the presence of EDTA was investigated. Results graphed in Fig. 4 were obtained from the same preparation as that which was used for the results shown in Fig. 2. In each case the same solutions in the same concentrations were used. Optical density measurements were made at room temperature and ATPase determinations at $37°$. Otherwise the only difference between Fig. 2 and Fig. 4 is that the former relates to ATPase activity, the latter to optical density changes. Mg^{++} and EDTA obviously influence the optical density effect in exactly the same way as they influence the enzyme activity, little change occurring until the Mg^{++} concentration exceeds the EDTA. Although the precise form of the curves in Fig. 4 may be in some doubt, the general conclusion is inescapable.

Further evidence for the connection between optical density changes and ATPase activities was provided by the use of inhibitors. Lack of opportunity prevented these experiments being widened in their scope, but Fig. 5 shows the effect of three different salts on the enzyme activity. NaF ($10^{-2}M$) caused more than 50% inhibition of the enzyme, and in the same concentration NaCl and NaCN had little effect. On increasing the salt concentration to $10^{-1}M$ the fluoride

inhibition was increased only slightly, but cyanide became very effective. It was found that whilst $10^{-2}M$ NaF inhibited the optical density change, $10^{-2}M$ NaCl and $10^{-2}M$ NaCN had no effect. Unfortunately the experiment could not be rounded off readily by making measurements in $10^{-1}M$ salt solutions since the salt itself, at this concentration, caused some slight optical density changes which confused the interpretation.

Measurements were always made within a few hours of isolation of the preparation. This was quite essential since five preparations which showed the optical density change in freshly prepared suspensions were quite inactive after storage overnight at $2°C$. Such storage resulted in no loss of enzyme activity, and therefore it would appear that this deterioration on storing was the result of some structural disintegration. Flagella and certain kinds of sperm tails are, of course,
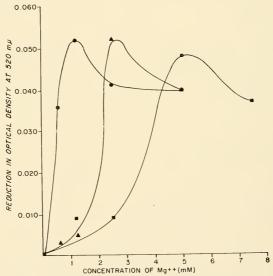
FIG. 4. Effect of Mg^{++} on the reduction in optical density at $520 \, m\mu$ obtained 20 min after addition of ATP to perch sperm tails (same preparation as illustrated in Fig. 2) in presence of EDTA. ●, no EDTA; ▲, 1.25 mM EDTA; ■, 2.5 mM EDTA.
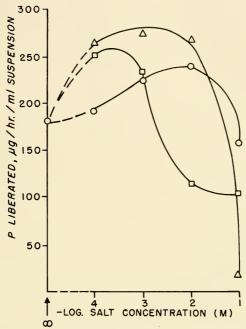
FIG. 5.   Effect of added salt on ATPase activity of perch sperm tails in absence of EDTA. Incubation at 37° for 10 min in pH 7.8, 0.05M tris buffer containing 0.5M sucrose, 2 mM ATP and 2.5 mM MgCl$_2$. ○, NaCl; △, NaCN; □, NaF.

well known for the ease with which they split into their component fibrils.

Several attempts were made to reverse the effect by adding excess EDTA to preparations in which the optical density change, taking place on addition of ATP and activator, had just been determined. These attempts were without success.

The general results indicate a reduction in optical density of some 15% usually occurs when the inhibited ATPase of perch sperm tail preparations is activated in the presence of substrate. Apparently ATP can only be replaced by ADP and the magnesium activator, to

some extent and rather ineffectively, by calcium. Otherwise the factors controlling the change are very specific. Moreover, the production of the effect in the presence of ATP follows closely those conditions governing the activation of the enzyme and there is little doubt that the optical density reduction and ATP splitting are intimately connected.

Presumably the optical density reduction is the result of a lowering in the refractive index of the sperm tails which take up water and swell. This is the interpretation given to this kind of result in the case of mitochondria by Cleland (1952) and would appear to be the only possible one in this case also. A direct confirmation of this could probably be obtained by comparing the wet weights of flagella obtained, on the one hand, from suspensions which have not been allowed to split ATP and, on the other, from suspensions which have been allowed to do so. This aspect of the matter is one with which the author is concerned at the present time.

The complete dependence of the process on ATP splitting makes it most unlikely that the change arises from a hemolysis-like leakage or from fragmentation of the tails. The effect could not arise as the result of the adsorption, with no volume change, of the substrate on the enzyme since, if this had any effect on the refractive index at all, it seems more likely that it would increase it. Moreover, overnight storage resulted in preparations which could still be activated enzymically and which would therefore still adsorb substrate and show any refractive index changes associated with this. After such storage however, these preparations did not show the optical density effect. This agrees with the swelling hypothesis and indicates that some kind of structural deterioration takes place on standing. This conclusion is also supported by the fact that not all freshly prepared sperm tails showed this effect. Yet in all these preparations the enzyme was capable of normal activation. Similarly, a swelling due merely to the introduction of water in the form of adsorbed and hydrated ATP ions should be latent in all preparations as long as the enzyme is capable of normal activation. Thus the swelling does not appear to be an artifact of ATP splitting but to have some special biological significance.

When the enzyme was inhibited in sperm tail preparations which had just finished swelling as the result of ATP splitting, no reversal of

the effect was obtained. This was disappointing, but it would appear that some stimulus other than simply the cessation of ATP splitting is necessary to restore the flagellum to its original size.

It is suggested therefore that the completely relaxed state in the case of sperm tails is one of minimum volume and is thus the opposite of the myofibril in this respect. Activation of all the sites of enzyme activity in the presence of substrate results in maximum swelling. Presumably between these two extremes is the state of the flagellum during motility where those enzymic sites which are hydrolyzing ATP cause localized swelling. Other sites prevented from splitting their substrate, possibly by the nonavailability of free ATP, do not show this swelling. The combination of substrate-splitting sites, which give rise to a corresponding tail configuration, is changing all the time.

Glycerol-extracted sperm and, in a few instances, glycerized sperm tails have been used as model systems (Bishop and Hoffmann-Berling, 1959). These models have provided and no doubt will continue to provide very useful information but for a study of the biomechanics of motility they suffer from two disadvantages. The presence of at least some midpiece components appears to be necessary before the models will flagellate (Bishop and Hoffmann-Berling, 1959), and ATP splitting takes place at some enzymic sites and is presumably absent at others. The very multiplicity of processes which must be taking place in these models makes the results difficult to interpret. Extracts of proteins involved in the mechanism have not proved easily obtainable (Bishop, 1958a), and in any case the behavior of such extracts is always difficult to relate to the behavior of the living material.

It is likely that the preparations described here will provide a useful model system which, in any case, will be complementary to the two mentioned above. Thus, if the biological process which accompanies ATP splitting has really been observed, and this seems to be the case, factors influencing this process can now be examined quite easily.

SPERM CELLS AND ACETYLCHOLINESTERASE

The generally accepted mechanism of nervous excitation requires the participation of the enzyme acetylcholinesterase. In the case of neuromuscular control this enzyme is probably also a constituent of

the muscle cell itself where it possibly exerts some intracellular control on ionic movements and thus the contraction of the myofibrils (Barrnett and Palade, 1959).

Sperm cells and protozoa in general are not, of course, innervated in the conventional sense and yet these organisms can show a very fine degree of control over their motility. In addition to enquiring into the actual mechanism of movement in these single cells, therefore, it is also pertinent to ask how this movement is controlled.

Other authors have made observations relevant to this problem. Thus, Seaman and Houlihan (1951) found acetylcholinesterase as a constituent enzyme of the ciliate *Tetrahymena* and identified the enzyme as associated with the pellicular fraction obtained from a cell homogenate (Seaman, 1951). In a brief note, Mann and Legge (Mann, 1954) mention the detection of the same enzyme in the tails of ram sperm but not in the heads. While Seaman (1955) suggests that the enzyme activity results from the presence of what he calls "subpellicular neurofibrils" the finding by Mann and Legge of acetylcholinesterase in organs which have a similar basic structure to that of cilia suggests Seaman's pellicular fragments may still have had cilia attached to them. On the other hand, while an acetylcholine mechanism might be located exclusively in the cell body of systems carrying simple cilia with only the basic pattern of two inner and nine outer fibrils, such a mechanism could well extend down or be located in the more complex tails of mammalian sperm. Attempts have been made at Leeds (Tibbs, 1960), first of all to confirm the presence of the enzyme in protozoa and then to decide whether or not the enzyme is associated with those basic structures of the cilium and flagellum which are common to all organs of this type.

No acetylcholinesterase could be detected in homogenates of the algae *Polytoma uvella* or *Polytomella caeca*. Precautions were taken to guard against enzyme inactivation and the presence of natural inhibitors, but homogenates of *Tetrahymena pyriformis* also proved to be inactive or, at least, to have an activity less than that which would split 0.01 μg of substrate/hr/mg at 37°. In this respect the author's results are at variance with those of Seaman and Houlihan (1951), but are in agreement with the results of yet another worker, Reith (1953), who has also been unable to demonstrate the presence of the enzyme in *Tetrahymena*.

With fish sperm (from *Salmo trutta* and *Perca fluviatilis*) the situa-

tion was rather different, and Table II shows that at $37°$ these sperma-
tozoa split acetylcholine to the extent of about 0.05 to 0.15 $\mu g/hr/mg$.
The splitting was inhibited by eserine, and this indicates that the en-
zyme is not a common esterase. The comparatively slight breakdown of
other choline and substituted choline esters also indicated that the
enzyme was a specific acetylcholinesterase and not a pseudocholinester-
ase.

TABLE II.   Hydrolysis of choline esters by fish spermatozoa at $37°$

Specimen[a]	Acetylcho-line Chloride Split ($\mu g/hr/mg$)	Acetylcho-line Chloride Split in $10^{-5}M$ Eserine ($\mu g/hr/mg$)	Butyrylcho-line Chloride Split ($\mu g/hr/mg$)	Acetyl-β-methylcho-line Chloride Split ($\mu g/hr/mg$)	Benzoylcho-line Chloride Split ($\mu g/hr/mg$)
1T	0.04				
5T	0.03				
6T	0.00				
8T	0.04		0.02		
9T	0.04		0.02		
10T	0.02		0.02		
11T	0.03	0.01	0.00	0.01	
12T	0.03	0.01		0.02	0.00
13T	0.03	0.01	0.01	0.01	0.00
14T	0.02	0.01	0.01	0.01	0.01
15T	0.02				
16T	0.00				
16T[b]	0.06				
17T	0.00				
17T[b]	0.10				
1P	0.10				
2P	0.13				
3P	0.08				
4P	0.05				
5P	0.15		0.01	0.05	0.02
6P	0.13		0.03	0.02	0.03
7P	0.08	0.00			
8P	0.11	0.00	0.01		
11P	0.06	0.01	0.03	0.05	0.07
12P	0.09	0.00	0.00	0.06	0.03
13P	0.09	0.00	0.00	0.01	0.03

[a] T, trout spermatozoa; P, perch spermatozoa.

[b] These preparations were from unwashed sperm, the activity of the plasma being
allowed for.

The most important part of this work was the determination of enzymic distribution. A suspension of perch sperm was shaken to break off the tails from the heads, and the resulting suspension was separated by centrifugation, as quantitatively as possible, into three fractions—heads, tails, and final supernatant. These fractions were made up to equal volumes as was an equivalent aliquot of the complete suspension.

Table III shows the distribution of the enzyme between the various fractions, the figures relating to activity per unit volume. It will be seen that the activity was concentrated in the head fraction, the contributions by the other fractions being accountable for on the basis of contamination with head material and the tendency of the enzyme to be extracted into the final supernatant.

It appears therefore that with spermatozoa possessing tails with the simple and unembroidered system of $(9 + 2)$ fibrils the acetylcholinesterase is located in the head. The apparent absence of the enzyme from the other ciliates described may only mean, of course, a low level of activity in these organisms.

Histological methods for the location of the site of acetylcholinesterase activity by the electron microscope are now becoming available (Barrnett and Palade, 1959; Lehrer and Ornstein, 1959), and since there seems to be a possibility that this enzyme may be found in the mammalian sperm tail but not as a component of the inner $(9 + 2)$ system of fibrils, the application of these histological methods to this type of tail may prove to be of considerable interest.

TABLE III. The distribution of acetylcholinesterase in homogenates of perch spermatozoa. Figures show acetylcholine chloride split in $\mu g/hr/ml$ at 37°

Specimen No.	Total Homogenate	Cell Heads	Cell Tails	Supernatant
1P	2.0	1.8	0.2	0.3
2P	3.9	1.3	0.6	
3P	1.7	1.3	0.0	
4P	2.0	2.0	0.2	0.0
6P	3.0	2.5	0.4	0.2
7P	2.9	2.3	0.2	0.5
8P	3.6	3.0	0.0	1.8
13P	2.2	2.5	0.2	

Glycerol and digitonin extraction would destroy a control mechanism based on permeability changes resulting from acetylcholine release and breakdown; Bishop (1958b) has, in fact, observed that, although his sperm models still flagellated on addition of ATP, these waves of flagellation did not progress down the tail and were uncontrolled. As a result, little translatory motion occurred.

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Reactivation of Extracted Sperm Cell Models in Relation to the Mechanism of Motility

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The striking parallel in recent methods of analysis of sperm motility and muscular contraction is not coincidental. For well over fifty years, attention has been directed toward the fibrillar nature of the sperm tail and the analogy of flagellar activity with movement in muscular systems, as testified by the statements of Ballowitz, Koltzoff, Ciaccio, Heidenhain, and other classical microscopists at the turn of the century (Bishop, 1962, for review). That studies of muscle took a commanding lead is a tribute to the brilliance and ingenuity of many able investigators, as well as to the size, distribution, characteristics, and importance of the chosen material. That studies of spermatozoa are making belated and sometimes plodding progress is evidence that movement, "contractile movement," is becoming recognized as a universal process and some advantage is to be gained by investigation of a system which may be less specialized, but is phylogenetically more ancient, than the exquisitely refined apparatus in, for example, the psoas muscle of the rabbit.

Investigations of muscular contraction have moved on a three-pronged front. The physiological background has been provided particularly by the elegant experiments of A. V. Hill and colleagues which have defined the velocity-tension-work parameters within which the contractile system operates (e.g., Gasser and Hill, 1924; Hill, 1938, 1949, 1950, 1958). At the other biochemical extreme, Engelhardt (1958), Szent-Györgyi (1951), Weber (1958), and many others stimulated by them have isolated and characterized contractile proteins from muscle fibers and have on occasion put them back. A

middle ground has been explored by leaving the contractile proteins in the fibers but extracting soluble substances with the result that a dead but contractile system is available for study in a relatively naked state. Szent-Györgyi (1949) and Varga (1950) first demonstrated the feasibility of preparing these glycerol-extracted models of muscle-fiber bundles, and the Webers (1950) adapted the technique to single muscle fibers. The preparation of extruded actomyosin thread models and compressed monolayer actomyosin models was soon perfected by Portzehl and Weber (1950) and Hayashi (1952). It is only fair to add that, despite giant strides made in these areas of muscle research, plus equally impressive advances in ultrastructural studies (see Huxley, 1957), a unifying theory of muscular contraction, involving activation, energy transfer, and mechanocoupling, has not yet been devised (Wilkie, 1954; Pringle, 1960).

Sperm motility studies likewise have, in large measure, advanced on a triple front. Although necessarily less precise, the physiology of flagellation has been defined in terms of velocity, amplitude, frequency, and power requirements in investigations initiated by Sir James Gray (1931, 1955, 1958) and discussed by others during this symposium. Significantly lacking, however, are data concerning changes in action potential or other detectable charge characteristics of spermatozoa during the flagellation cycle. On the other hand, the extraction of musclelike sperm adenosine triphosphatases (ATPases) is an established fact and, while the enzymes' characteristics and vital role have been only partially elucidated, they have been immortalized, in one species at least, by virtue of the distinction of having been named "spermosin" (Engelhardt, 1946). While essentially like myosin in respect to many properties (Bishop, 1962), the extractable sperm contractile proteins, at least of lower vertebrates, behave like actomyosin-type proteins (see Pautard, this symposium). An actinlike moiety has reportedly been isolated from sea urchin spermatozoa by A. Ruby (personal communication). The third alternative procedural pathway in the study of sperm motility, the preparation and activation of flagellar models, is the one to be pursued here, since the results seem relatively clear-cut even though the interpretations may be limited.

Parenthetically, the designation of these preparations as *models* is consistent with the term as used by Szent-Györgyi and others in re-

ferring to glycerol-extracted muscle preparations. Perhaps a more correct term would be reconstituted cells or fibers as suggested by Hayashi. Nevertheless, the distinction should be clear between extracted sperm cell models as described here and the conceptual models of contraction envisaged by Pringle (1960), for example, or the actual mechanical models of spermatozoa fabricated by Sir Geoffrey Taylor (1952).

To Hoffmann-Berling (1955a) goes the credit for having first applied the extracted cell procedure to nonmuscular systems, including spermatozoa. Glycerol-KCl-extracted sperm of the locust, Tachycines, were found, after storage, to respond to adenosine triphosphate (ATP), in the proper ionic milieu, by assuming a vigorous and continuing rhythmic motility. Since that time, ATP-sensitive model systems have been described for a variety of flagella, cilia, interkinetic and mitotic fibroblasts, sarcoma cells, vorticellid myonemes, paramecium trichocysts and T_2 bacteriophage (Hoffmann-Berling, 1955b, 1958a, b, 1959; Brokaw, 1961; Alexandrov and Arronet, 1956; Levine, 1956; Kinoshita, 1958, 1959; Kozloff and Lute, 1959; Bishop and Hoffmann-Berling, 1959).

Since the methods of extraction of sperm models have been adequately presented elsewhere (Hoffmann-Berling, 1955a,b; Kinoshita, 1958; Bishop, 1958a; Bishop and Hoffmann-Berling, 1959), the subject may well be omitted here. Attention should be directed, however, to the possible role and significance of glycerol in the actual extraction process. In order that sperm models may be preserved in a reactivatable state at subzero temperatures, they must be bathed in an approximately 50% glycerol-saline medium, comparable to the muscle extraction medium advocated by Szent-Györgyi. Mammalian spermatozoa, however, can be satisfactorily extracted at 20°C without glycerol and subsequently reactivated with ATP (Bishop and Hoffmann-Berling, 1959). Studies on these cells indicate that glycerol is more important in the stabilization and preservation of the contractile protein system than in the extraction process per se.

In order to ensure complete extraction, certain criteria were established for these preparations. When properly treated, extracted mammalian sperm showed neither respiratory activity (oxygen consumption) nor glycolysis (lactate formation); reactivation was inducible only by nucleotides at reasonable concentrations; deteriora-

tion of permeability and ionic balance was further indicated by failure of wave propagation along the flagellum and a consequent lack of forward progression of the reactivated cells (Bishop and Hoffmann-Berling, 1959). In the latter respect, sperm models differ, perhaps as a result of more complete extraction, from the models of Polytoma flagella discussed by Brokaw elsewhere in this symposium.

The salient and truly spectacular feature of sperm model reactivation is the fact that the addition of "physiological" amounts of ATP to the system initiates rhythmic wave formation which in bull sperm models may persist for two hours or more. This is in striking contrast to muscle models which ordinarily, upon the addition of ATP, shorten once and remain in a contracted state until external conditions are altered. In flagellar models both contractile and relaxation phases—the "shortening" and "elongation" processes—of the bending cycle are set in motion by ATP, and the perpetuation of the rhythm suggests an oscillating mechanism rather than a simple contraction-relaxation system.

Both amplitude and frequency of beat following reactivation prove to be in many, if not most, preparations quite comparable to that of live sperm. The ATP-induced bending wave of bull sperm, for example, shows a maximal amplitude of about 10 microns, approximately twenty times the tail diameter and far exceeding that which might be anticipated if nonspecific forces such as Bronwnian movement were responsible for motility. Moreover, the shape of the induced waves compares favorably with the major uniplanar bending cycles of normal sperm in that both sides of the flagellum are active and capable of what appears to be contraction. Wave formation may occur throughout the flagellum, or be limited to a restricted proximal and/or distal segment; there is no indication in the sperm models that wave formation begins at the base and is propagated toward the tip of the flagellum. Three-dimensional wave motion is apparently absent and thus there is no helical movement or rotation along the longitudinal axis of the flagellum.

Whereas the capacity for wave formation is retained by the extracted cells, coordination and wave propagation are completely destroyed. Along with this loss, the capacity for forward progression likewise disappears (Hoffmann-Berling, 1955a; Bishop and Hoffman-Berling, 1959). No adjustment of ionic balance has been found which

restores the normal proclivity for wave *initiation* at the base of the tail and its subsequent *propagation* along the flagellum.

ATP is dephosphorylated by sperm models but, on a cell basis, at a very low rate—on the order of 0.2 μg P liberated per milligram N per minute by bull sperm. Substrate specificity is not limited to ATP since adenosine diphosphate (ADP) and inosine triphosphate (ITP) are also utilized by mammalian sperm models (Bishop and Hoffman-Berling, 1959). ATP is effective on these models over a range of about 0.1 to 10 mM; ITP requires a higher minimum concentration. ADP appears to be split, but is probably first converted to ATP and AMP by an adenylatekinase which is present in briefly stored sperm models but which appears to be leached out with continued extraction and storage.

The rate of flagellation of sperm models of some species varies with changes in concentration of nucleotide. The response of sea urchin and starfish sperm models, studied by Kinoshita (1958), showed a maximal frequency at 1 mM ATP (Fig. 1). Above a supraoptimal limit of about 10 mM, ATP was ineffective in inducing motility. This closely corresponds to the supraoptimal concentration of ATP for isolated muscle systems (Weber and Portzehl, 1954). Two interrelated causes may be suggested for the failure of ATP in high concentration to induce flagellation: substrate inhibition of the splitting enzyme and the plasticizing action of unsplit nucleotide. A curvilinear relation between ATP concentration and frequency of response of intact sperm models is shown in Fig. 2. Originally portrayed as a direct effect of splitting activity, the increase in frequency of beat was found to result from the plasticizing action of ATP, since at a constant concentration of nucleotide, the same curvilinear relation occurs with increasing concentrations of inorganic pyrophosphate (Hoffmann-Berling, 1955a). A change in ionic strength also increases the rate of flagellation, presumably by a "softening" effect (Fig. 2). In most mammalian sperm, on the other hand, no increase in frequency occurs with increasing ATP concentration; the flagellation rate remains constant over the concentration range (Bishop and Hoffmann-Berling, 1959). Possibly this type of response is attributable to the presence of adequate plasticizing capacity of extracted mammalian sperm not found in the invertebrate models.

The rate of induced flagellation is temperature-dependent and
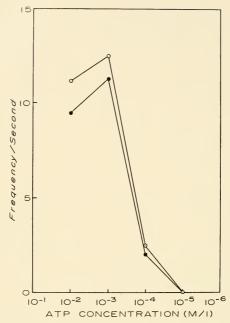
Fig. 1. Effect of ATP concentration on rate of flagellation of sperm
models of sea urchin (○) and starfish (●), in presence of 5 mM Mg++
and 10 mM histidine at pH 7.5. (After Kinoshita, 1958.)

shows a Q_{10} value approximating 2 over the range from 0° to 20°C
(Fig. 3). These data for insect sperm were obtained from cinephoto-
micrographic records (Hoffmann-Berling, 1955a). Mammalian sperm
models are inactivated at a temperature of about 27°C; sperm models
of the invertebrate squid (Loligo) fail to respond above 12°C (Bishop,
1958a; cf. Matoltsy, 1950).

The pH range for reactivation is fairly broad and could reflect the
unusual tolerance of many species of living sperm toward acid-alkali
changes (Mann, 1954; Bishop, 1961). Bull sperm models, for example,
respond to ATP over a range from pH 6.5 to 8.0 or greater, with an
optimum at 7.2 to 7.4. The frequency of beat of sea urchin and star-
fish spermatozoa is plotted against pH in Fig. 4; variation from 7.2
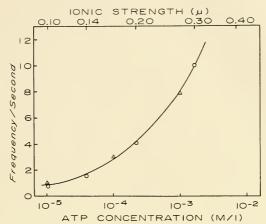
Fig. 2.   Variation in frequency of insect sperm models as a function of ATP concentration ($\triangle$) and of ionic strength ($\bigcirc$). (After Hoffmann-Berling, 1955b, 1959).
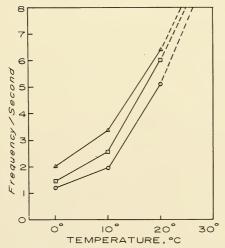
Fig. 3.   Temperature dependence of insect flagellar models reactivated with 1 mM ATP. $Q_{10} \simeq 2$. (After Hoffmann-Berling, 1955a.)
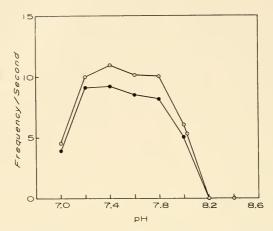
FIG. 4.   Effect of pH on beat frequency of models of sperm of sea urchin
(○) and starfish (●), reactivated with 1 mM ATP, 5 mM Mg^{++} and 10
mM histidine. (After Kinoshita, 1958.)

to 7.8 causes no significant change in flagellar rate, as determined
visually by ordinary microscopic methods (Kinoshita, 1958).

In studies of cationic requirements, Mg^{++} proves essential for re-
activation of sperm models of both invertebrate and vertebrate species
(Hoffmann-Berling, 1955a; Kinoshita, 1958; Bishop and Hoffmann-
Berling, 1959); Ca^{++}, on the other hand, while not required in the
bathing medium, facilitates reactivation at least of mammalian sperm,
possibly by virtue of an effect on plasticity or "relaxation" of the
cells (see below). It is to be noted that divalent cations, particularly
Mg^{++}, are probably bound and are not entirely removed by extrac-
tion; thus, absolute requirements are difficult to quantitate. Kinoshita
(1958) demonstrated an optimal exogenous Mg^{++} concentration of
10 mM for models of sea urchin and starfish sperm (Fig. 5). These cells
were reactivated by ATP in the presence of histidine, which has a
chelating action, and the concentrations as indicated should be re-
garded with this in mind. In bull sperm models the concentrations
of Mg^{++} in both extraction and reactivation media influence the
time required for reinitiation of motility by ATP (Table I).

K$^+$ and Na$^+$ are completely interchangeable in this system, and
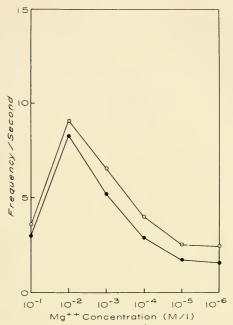
Fig. 5. Frequency as a function of Mg^{++} concentration in sea urchin (○) and starfish (●) sperm models; 1 mM ATP, 10 mM histidine, pH 7.5. (After Kinoshita, 1958.)
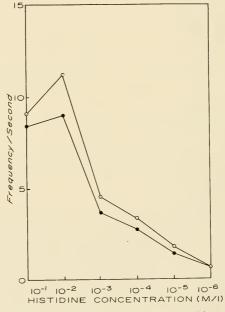
both ions sustain flagellation equally well. A similar monovalent cationic indifference was noted by Nelson (1955) in his study of ATPase activity of clam flagella and is consistent with evidence for ionic requirements of comparable muscle preparations (Szent-György, 1951).

Whereas reactivation of insect and mammalian sperm models requires only exogenous nucleotide and Mg^{++}, starfish and sea urchin sperm need an additional factor, a plasticizing or chelating agent such as histidine, cysteine, EDTA, or pyrophosphate (Fig. 6.) (Kinoshita, 1958). This suggests that a relaxing type of substance is essential to the invertebrate sperm model as extracted; such an agent, along with ATP, is necessary for oscillation. Kinoshita (1959) subsequently iso-

TABLE I. Effect of magnesium concentration on initiation of ATP-induced motility in bull sperm models[a]

| Mg^{++} Molar Concentration | | Reaction Time (seconds) |
Extraction Medium	Reactivation Medium	
6×10^{-3}	4×10^{-3}	$<2-10$
4×10^{-3}	4×10^{-3}	$15-30$
2×10^{-3}	4×10^{-3}	$45-70$
None added	4×10^{-3}	$56-188$
4×10^{-3}	2×10^{-3}	>110

[a] Data from Bishop and Hoffmann-Berling, 1959.

FIG. 6. Flagellation rate of sperm models of sea urchin (○) and starfish (●) at different concentrations of histidine; 1 mM ATP, 5 mM Mg^{++}, pH 7.5. (After Kinoshita, 1958.)

lated from starfish sperm a relaxing substance by the Bendall (1954) procedure for the extraction of Marsh factor from striated muscle (Marsh, 1952). This component, in the presence of ATP and Mg^{++}, effects reactivation of invertebrate sperm models and also, interestingly enough, brings about relaxation of shortened frog muscle (Kinoshita, 1959). Similarly, extract of relaxing factor from frog muscle is effective in the sperm reactivation system, and Kinoshita regards the two factors functionally, if not chemically, identical (see Tibbs, this symposium).

Kinoshita's findings on invertebrate sperm are consistent with our own view that something akin to "relaxing factor" is of general significance in the operation of mammalian sperm models. If, for example, Marsh-Bendall factor, extracted from the rabbit psoas muscle (Bendall, 1953), is added to bull sperm models in the presence of ATP and Mg^{++}, no reactivation occurs (Bishop, 1958b). The addition of Ca^{++}, however, one effect of which is to block Marsh-Bendall factor activity, results in immediate flagellation (Table II). Ca^{++} alone has no such inducing effect on the system. Treatment of relaxing factor with Ca^{++} prior to its addition to the models drastically reduces its inhibitory activity (Bishop and Hoffmann-Berling, 1959). This Ca^{++}-dependent activity of muscle relaxing factor could not be mimicked by nonspecific plasticizers such as EDTA or inorganic pyrophosphate in mammalian sperm models (cf. Nelson, 1959). Whether mammalian spermatozoa contain a relaxation substance identical with that reported in invertebrate sperm by Kinoshita is not known, but they do appear to possess some component which binds Ca^{++} readily and has

TABLE II.   Responses of bull sperm models to Marsh-Bendall factor extracted from rabbit skeletal muscle[a]

Additions to Extracted Sperm	Responses
ATP $10^{-3}M$	+
Ca^{++} $10^{-2}M$	−
ATP $10^{-3}M$ + Ca^{++} $10^{-3}M$	+
ATP $10^{-3}M$ + MBF − Ca^{++}	−
+ Ca^{++} $10^{-3}M$	+
ATP $10^{-3}M$ + MBF − Ca^{++}	−
+ Mg^{++} $5 \times 10^{-3}M$	−

[a] Data from Bishop, 1958b; Bishop and Hoffmann-Berling, 1959.

a plasticizing action similar to that of muscle relaxing factor. This component is not washed out during extraction or lost during storage as seems to be the case with Kinoshita's relaxing factor of invertebrate sperm. It is unlikely that this agent is adenylatekinase (myokinase) since the latter activity is lost during prolonged extraction and storage of mammalian spermatozoa.

Two further physical characteristics of the model system may be noted. Reactivated sperm can flagellate against an imposing viscous stress exerted by an increase in the viscosity of the surrounding medium (Bishop and Hoffmann-Berling, 1959; Brokaw, this symposium). Attempts to assess the capacity of mammalian sperm models to exert a "maximal tension" equivalent to that of living cells were only partially successful; nevertheless, the experiments did demonstrate that mechanical work can be done by the reactivated cells against an increased external force.

Isolated flagella and fragments of the tails of mammalian sperm models were prepared and tested for reactivation ability. Only intact tails responded to ATP; distal fragments, even large segments, failed to respond (Bishop and Hoffmann-Berling, 1959). This finding would seem to support the view that the basal body and perhaps even the mitochondrial region of the flagellum are essential for induced movement, indicative of a whiplash type of process. The response of individual segments of the intact flagellum, however, suggests that cutting the tail into fragments severs long-chain constituents or filaments necessary for shortening, similar to the cutting of an origin or insertion of a muscle fiber.

What bearing have such findings on the problem of the mechanism of sperm movement? Interpreted in terms of muscle biochemistry, some light is shed upon the contractile protein nature of the system, on its nucleotide and cationic dependence, on the operation of an oscillating process rather than on independent and discrete contraction and relaxation events. But beyond that, the use of the model system as an experimental tool also affords an insight into associated phenomena of flagellation at a cellular or physiological level. By the very isolation of the functional wave-forming system, other aspects of normal motility which are altered or abrogated by the extraction process are more clearly delineated or set apart.

As a result, for example, of what would seem to be the destruction of normal permeability characteristics and ionic balance brought about by extraction, the physiological properties involving coordination and intrinsic irritability of sperm models are lost. An acetylcholine-choline esterase system may also be destroyed (Tibbs, this symposium). Coincident with these changes, two wave characteristics are sacrificed: initiation at the base of the flagellum of the major uniplanar wave, and its propagation toward the tip. The former property suggests that normally some kind of stimulating mechanism, or pacemaker, may reside at the flagellar root, in or near the basal granule from which the axial filaments arise. This stimulus for wave formation could conceivably originate in pulsed action potentials or potential discharges, or be generated by rhythmic metabolic changes possibly arising in, for example, the mitochondrial apparatus. Such a concept of wave stimulation is not new (cf. Astbury *et al.*, 1955; Bradfield, 1955), but the idea persists and is underscored by sperm model studies which set this off as an event separate from wave formation per se. The demonstration and elucidation of a stimulating and coordinating system in sperm must depend on the skillful acquisition of precise and pertinent data which may relate intracellular electrical changes to activity during the flagellation cycle.

The abolition of wave propagation in the models further implies that a coordination mechanism is normally at work which involves some kind of orderly transmission. Suggestions as to the basis for such propagation have ranged from undesignated membranes and "conductile" filaments (Bradfield, 1955; Inoué, 1959) to the contractile elements themselves (Machin, 1958; Pautard, this symposium). The failure of sperm models to propagate the active bending couples that do occur suggests that there may very well be conductile characteristics in normal flagella which are destroyed by extraction and permeability changes (Bishop, 1958c).

Judging from these flagellar models, the major or predominant wave pattern—the only one found in extracted sperm—is two-dimensional. The three-dimensional component which gives rise to helical movement and which we believe is characteristic of motility of healthy live sperm (Bishop, 1958c) is absent. This might be attributable to the failure of wave propagation in the models. We feel, however, that

it is more closely associated with the loss of a coordinating system, not necessarily involving overt wave propagation, since one can demonstrate that three-dimensional tail movements and cell rotation can occur in the absence of wave propagation in, for example, live sperm of the squid when exposed to less than favorable conditions (Bishop, 1958c), a point raised by Lord Rothschild during this conference. Propagation of the wave may not be essential for flagellar activity in more than one plane; propagation certainly is required, however, to effect forward progressive movement of the cell.

Induced flagellation in sperm models is most simply to be regarded as the result of priming a self-sustaining oscillating system, a view consonant with that expressed by others in this symposium (cf. Pautard, Brokaw). It is tempting to compare the mechanism of flagellar activity with that of any one of several unique systems of transiently oscillating muscle preparations (Goodall, 1956; Lorand and Moos, 1956; Pringle, 1957), but such comparisons at this time seem based on rather fragile evidence. If a common denominator of cellular movement can be found here, all well and good; on the other hand, it is not inappropriate to recall the work of Kuhn et al. (1959), which demonstrated that all that contracts need not be muscle.

The characteristics of the response of sperm models to nucleotide has provided compelling evidence that (1) the energy transfer which permits chemomechanical coupling is locally distributed along the flagellum, and (2) wave formation is a local or segmental affair and is not brought about by a whiplash mechanism restricted to the base of the tail. This applies to the extracted cells, and we regard it as equally cogent in the flagellation mechanism of living spermatozoa.

Regardless of which components within the flagellum prove to be the motile elements—be they the longitudinal filaments or some other, as yet unrecognized, units—it is difficult to conceive of a mechanism which does not demand simultaneous "shortening" and "elongation" at any given section of the tail. Even the highly speculative suggestion of a sliding-filament theory for sperm movement (Afzelius, 1959) would require overlapping on one side and stringing out on the other. Contraction, if in the literal sense it does occur, must then *accompany* relaxation; likewise, as any one motile element passes from the contractile to the relaxing phase, its counterpart must

make the reciprocal change. The situation parallels that of a pair of flexor-extensor muscle fibers inseparably fixed in opposition to one another.

This coupled process makes it difficult to assign a precise role of ATP, for example, to the flagellation cycle. Aside from the question whether the nucleotide is bound or split during the active phase, the problem concerning the exact time when ATP exerts its effect cannot be simply resolved (Weber, 1958; Hoffmann-Berling, 1959). On a priori grounds, we may assume this to occur during active contraction or shortening of motile elements of the sperm flagellum, but Hoffmann-Berling (1958a) has shown that in at least one oscillating system, the vorticellid myoneme, ATP activity is associated with elongation and Ca^{++} excess with contraction (see also Tibbs, this symposium).

Further assumptions along these lines suggest the interesting possibility that ATP might serve as both contracting and relaxing substance depending on its rate of splitting, on the one hand, and excessive accumulation, on the other. On a basis of the studies reported here, it seems more likely that nucleotide activity represents part of the cycle and is balanced against a component or process which effects the completion of the oscillation. Dr. Nelson has presented evidence during this symposium which suggests that the contractile phase is dominated by ATPase activity and the relaxation phase by IPPase action. The evidence from our models indicates that a relaxing substance or factor, possibly unique to these cells but replaceable by muscle relaxing factor, is present which counterbalances the ATPase complex. One way in which such a system might operate is by the interchange and momentary binding of the cations, Ca^{++} and Mg^{++}, with the two components during the oscillation cycle. During contraction or shortening, Mg^{++} is bound to the ATPase system and Ca^{++} is bound to and inhibits the relaxing system; during elongation the affinities of the cations are reversed. A much more rapid shift of Ca^{++} and Mg^{++} over the distances that might be here involved has been envisaged by Csapo and Suzuki (1957) for the excitation-contraction coupling of muscle fibers. The forces which evoke these ionic migrations and the velocities of response certainly differ in these two systems. Nevertheless, the concept of the regulation of

oscillation by ionic shifts and binding seems to be worthy of further experimental investigation.

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Studies on Isolated Flagella*

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At the present state of our knowledge, there is little reason to divorce the problem of spermatozoan motility from the general problem posed by the rhythmic, propagated bending waves found in all types of flagella. In approaching this general problem, the flagella of the orange, biflagellate alga, *Polytoma uvella*, offer some advantages. The algae can be grown in quantity in any geographical location and at any time of the year. Their flagella have an uncomplicated ultrastructure similar to that of the simplest appearing spermatozoa, such as those of the sea urchin. There is a clear distinction between the flagella and the remainder of the cell which supplies the flagella with sources of chemical energy, so that simple techniques have been developed for preparing clean fractions of isolated flagella. One of these isolation techniques (Brokaw, 1961) involves a relatively gentle treatment similar to that introduced by Hoffmann-Berling (1955) for the preparation of sperm models. The isolated flagella can be stored in glycerol solutions at $-20°C$ and can then be reactivated to a high degree when placed in appropriate solutions containing ATP. With flagella prepared in this way, biochemical studies and studies of movement can be carried out on the same material under the same conditions.

The limited amount of biochemical work carried out with this material to date has been described elsewhere (Brokaw, 1961) and will only be summarized briefly before turning to a consideration of some aspects of the movement of the isolated flagella. The ATPase activity of *Polytoma* flagella was first demonstrated by Tibbs (1957), using another type of preparation. With preparations containing intact fla-

gella, which can be counted, the ATPase activity can be expressed on a per flagellum basis, so that it can be more directly compared with calculations of power requirements. The Michaelis-Menten constant for ATP dephosphorylation by flagella is in the neighborhood of $10^{-5}M$, providing a further bit of evidence for biochemical similarity between flagella and muscle.

The flagella will also beat and liberate inorganic phosphate when placed in solutions containing ADP. Chromatographic indentification of ATP after incubating flagella with ADP demonstrated that they possess an enzyme analogous to the myokinase of muscle, which converts 2 ADP to ATP + AMP.

Flagella from *Chlamydomonas moewusii* can also be reactivated and have about as much ATPase activity per flagellum as *Polytoma* flagella. However, flagella isolated from one of Lewin's "paralyzed" mutants of *Chlamydomonas* could not be activated by ATP, and possessed only about one-third the ATPase activity found with normal flagella (Brokaw, 1960). Not much can be made of this isolated result, but it does suggest another channel of approach to the problem of flagellar motility.

For observations of motility, the flagella suspensions are diluted with 10 volumes of a solution containing ATP, $0.004M$ $MgCl_2$, $0.05M$ KCl, $0.02M$ tris-thioglycolate buffer, pH 7.8, and 1% polyvinylpyrrolidinone. Motility can be observed at room temperatures, but lasts much longer at $10–14°C$. The movement usually stops when the flagella become stuck down to the surface of the slide or cover glass, but if this does not happen, the flagella may beat for up to two hours.

At first, most of the isolated flagella are motile, and swim regularly through the suspending medium, basal end first. Forward progression is accompanied by rotation around a longitudinal axis, with about one revolution for three to five beats. Many of the flagella become attached to the slide or cover glass only at their basal end, so that their beating can be observed under simpler conditions without the complication of rotation. When observed with stroboscopic illumination at a frequency slightly less than the frequency of beat, the propagation of contractile waves along the flagella can be seen clearly. The direction of wave propagation is always normal—from base to tip, and the waves appear to be primarily planar.

The statement that isolated flagella can swim around by them-

selves may be a bit hard to believe. Unfortunately a satisfactory photographic demonstration of this swimming is still not available. However, wave propagation, which is the prerequisite for forward progression, can be demonstrated with simpler photographic equipment, and this is being attempted. Preliminary results, sampled in Fig. 1, are technically of low quality, but nevertheless show that the beating does not have a standing wave pattern, but is propagated along the flagellum.

In general, the beating of the reactivated isolated flagella appears qualitatively similar to that of normal flagella. It shows the three features of normal beating—wave propagation, rotation, and forward progression—commonly absent in reactivated mammalian spermatozoa (Bishop and Hoffmann-Berling, 1959), possibly because a relatively mild "extraction" technique is satisfactory with these thin flagella.

Quantitatively, the degree of bending of the isolated flagella is less than normal. The amplitude of 2 to 3 microns is perhaps about one-half the normal amplitude, and the wavelength is greater than normal. The swimming speed of isolated flagella varies around 10 microns/sec, compared with a swimming speed of 40 to 50 microns/ sec for normal cells at the same temperature. A rough calculation of the energy required to sustain the beating of isolated flagella, by using the equations of Taylor (1952), indicates that only about 5% of the energy available from dephosphorylation of ATP is being converted to movement against the viscous resistance of the medium. Most of the available energy would be needed to sustain the beating of flagella of normal cells.

Two possible interpretations of the apparent inefficiency of reactivated isolated flagella come to mind. The flagella may be abnormally stiff, on account of the preparative treatment, so that energy is required to overcome the internal resistance of the flagella as well as the viscosity of the medium. On the other hand, there may be a partial uncoupling between ATP dephosphorylation and movement, so that only a part of the available chemical energy can be transformed into mechanical work. The viscosity of the medium should have a relatively small effect on movement in the first case, and a much larger effect in the second case. Some results of experiments with media of increased viscosity are shown in Fig. 2.

The swimming speed of normal cells of *Polytoma uvella* was meas-
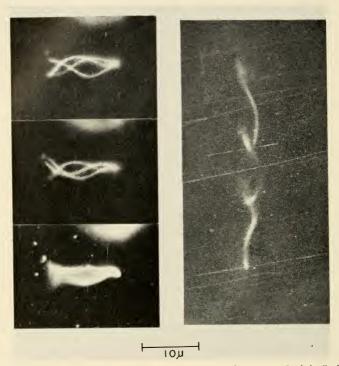
FIG. 1. Left, one-half second time exposure and two sets of triple flash exposures of an isolated flagellum attached to the slide at its basal end. Right, a triple flash exposure of an unattached isolated flagellum, showing forward progression. Interval between flashes is about 2 sec.

ured at 29°C by the dark-ground-track method (Brokaw, 1958). The swimming speed decreases markedly with viscosity, as expected if the cells are operating efficiently and using most of the available energy to move against the viscous resistance of the medium. The viscosities were varied with methyl cellulose and measured with a standard capillary tube viscometer. This represents a naive approach to viscosity, which may require modification on further study. In Fig. 2, viscosity is expressed on a logarithmic scale.
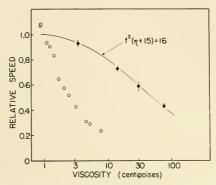
Fig. 2. Measurements of swimming speed of normal cells of *P. uvella* (○) and beat frequency of isolated flagella (●) in media of increased viscosity. η = viscosity of medium; f = beat frequency.

The frequency of beat of isolated flagella, determined at $11°C$ with a stroboscopic method, is very much less sensitive to viscosity changes than is the swimming speed of normal cells. This is consistent with, but by no means proves, the idea that the inefficiency of isolated flagella may be due to an abnormally high internal viscosity. In fact, the results of this and other experiments can be fit rather well by an equation of the form shown in Fig. 2, which was used to draw the line through the points representing measurements of beat frequency. There was no obvious change in amplitude or wavelength of beating in these experiments, but no quantitative measurements of amplitude have yet been attempted. If the amplitude is constant, the equation in Fig. 2 implies that the power available for flagellar activity is constant and is the factor which limits the frequency of beat as the viscosity of the medium is increased. If the equivalent internal viscosity of about 15 centipoises indicated by these results is taken into account, the calculated power consumption of the isolated flagella corresponds to use of about 2 or 3 kcal of energy for each mole of ATP dephosphorylated, which is a respectable efficiency.

The frequency of beat of isolated flagella also varies with ATP concentration, as shown in Fig. 3. These results closely resemble results obtained by other workers with glycerol-extracted sperm flagella, other than those of some mammals (see Bishop, this symposium). The
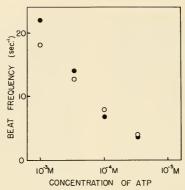
FIG. 3. Effect of ATP concentration on the beat frequency of isolated flagella (two preparations).

increase of beat frequency with ATP concentration has been attributed to a "plasticizing" effect of ATP. However, no significant discussion of how a plasticizing effect should be related to the parameters of flagellar activity has been presented. The ATP effect may be too valuable a clue to the mechanism of flagellar activity to be dismissed so readily. A plasticizing effect might mean that ATP decreases the internal viscosity of the flagella. This possibility can be examined by measuring the effect of viscosity on movement at different ATP concentrations. Some preliminary results are shown in Fig. 4.

The results presented in Fig. 4 should lie on straight lines if an equation of the form used in Fig. 2 is valid, and the intercept with the abscissa represents the value of the equivalent internal viscosity. If the action of ATP were a reduction in internal viscosity, a set of three lines of equal slope and different intercept should result. Instead, the values indicated for the internal viscosity are roughly the same at all three ATP concentrations. The major effect of ATP is a change in the slope of the lines, which implies that more energy is being made available to overcome internal and external viscous resistances, as ATP concentration is increased. The validity of this conclusion depends on the constancy of the amplitude of beat, which has not yet been accurately measured. Since the rate of ATP dephos-
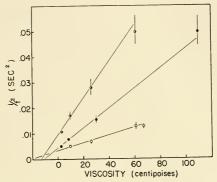
FIG. 4. Effect of viscosity on beat frequency (*f*) at ATP concentrations of $1 \times 10^{-4}M$ (◑), $3 \times 10^{-4}M$ (●) and $5 \times 10^{-4}M$ (○).

phorylation is independent of ATP concentration in this range, there is apparently a change in the efficiency of utilization of chemical energy provided by ATP dephosphorylation.

The work involving stroboscopic measurement of beat frequency was begun only recently, and only these few results have been obtained thus far. It is certainly premature to attempt to use them to test any theories of flagellar activity, but some discussion of theory is appropriate here. If we assume that some portions of flagella must convert chemical energy of ATP into mechanical work as expressed by active contraction, the remaining basic question is: How is the rhythmic, propagated contraction of flagella coordinated, even after isolation and treatments which are supposed to disrupt completely membrane permeability? One possible explanation has been suggested by the work of Boettiger, Pringle, and others on the rhythmic activity of insect fibrillar muscle (Pringle, 1957; Machin, 1958).

These muscles maintain oscillations at a frequency which is determined not by the frequency of membrane excitation, but by the mass and elasticity of the mechanically oscillatory system to which they are coupled. The amplitude of oscillation is determined by the resistive load on the system. The contractile element in the muscle can act as the equivalent of a negative resistance element, to supply power dissipated in the resistive load as the mechanical system oscil-

lates. These properties can be duplicated by a simple electronic ana-
log. The mechanism of oscillation can also be expressed by saying
that the active tension exerted by the muscle varies in such a way
that there is a constant time delay of about 7 msec between maximum
length and maximum tension (Machin and Pringle, 1960).

The extension of these ideas to flagella implies that the contrac-
tion of muscle-like elements distributed through the flagellum is
triggered by their extension by passive elasticity and the active con-
traction of antagonistic elements. There must be a time delay be-
tween length changes and changes in active tension. The result will
be a steady oscillation of each contractile element through a cycle of
relative deactivation, allowing extension, followed automatically by
relative activation leading to shortening. Twenty years ago the pro-
posal of a model of this complexity would have sounded wildly
speculative. Today it sounds considerably more reasonable, because
we have seen such spontaneous oscillations in insect flight muscle and
in some muscle model systems, because we have found some bio-
chemical similarities between flagella and muscle, and because we
have finally discarded the idea that the flagellum is a passive filament
waved by contractile elements situated in the basal granule. The
above model not only explains oscillation but also eliminates the
need for a separate mechanism, above the level of the contractile
elements, to produce a uniform propagated wave. The apparent co-
ordination can be explained simply by the sensitivity of individual
contractile elements to elongation by movement of other parts of
the flagellum.

The analogy between insect flight muscle and flagella cannot be
carried very far, since it has been generally accepted that the inertial
forces acting on a flagellum are negligible compared to the elastic
and viscous forces (Taylor, 1952; Gray, 1955; Machin, 1958). The
frequency and waveform of flagellar oscillation must be determined
by something other than the interaction between the mass and elas-
ticity of the system. The effect of increased viscosity on frequency
rather than on amplitude of flagellar oscillation also cannot be ex-
plained with reference to a typical mechanical oscillator. In flagella,
oscillation must involve some more intimate interaction between the
biochemistry of the contractile elements and the mechanical parame-
ters of the flagellum. Correspondingly, a much more complicated

electronic analog appears to be required to simulate the behavior of the flagellar oscillator.

We might also suggest that in the case of flagella, where beat frequency is not completely determined by mechanical parameters, there is a more direct relationship between the frequency and the time lag between length and active tension. The time delay might originate in a biochemical process which is sensitive to ATP concentration and other chemical factors. For effective beating, the peak tension exerted by the contractile elements should come in phase with the sum of the elastic and viscous resistive forces. The phase angle of this sum will vary with viscosity. If the viscosity is low enough, the resistance will be primarily elastic and will be maximal at 180° past maximum elongation. In high viscosity, the maximum resistance will occur 90° past maximum elongation (Machin, 1958).

This analysis implies that if there is a constant time delay between length and tension, and if this delay determines the frequency, then it would not be possible for the frequency to be varied by more than a factor of 2 by changing the viscosity of the medium, so long as the amplitude and waveform remain constant. This conclusion is certainly not supported by the film Lord Rothschild has shown us of bull spermatozoa swimming in a medium of high viscosity, but preliminary observations on isolated *Polytoma* flagella do give some indication of aberrant movement at very high viscosities. This point requires further examination; these experiments may provide some basis for comparing the behavior of flagella with that of the insect flight muscle system. The conclusion which begins to emerge from these preliminary experiments is that the basic idea that flagella contain contractile elements sensitive to elongation may be valid, but that the details of the feedback between length and tension may be peculiar to flagella, with little carry-over from investigations of insect fibrillar muscle.

In discussing these models, a number of problems have been ignored, such as: Why are contractile waves propagated in only one direction? Do the isolated flagella retain any morphologically differentiated region at their basal ends which may play a part in their motility? Why is the wavelength of flagellar beating independent of viscosity and ATP concentration but strongly correlated with the length of the flagellum? How does ATP reach the isolated flagella?

Is is entering through the broken end, or will the system work equally well if it is not bounded by any permeability barrier? Finally, the ultimate question: Are there any functional reasons for the uniformity of flagellar ultrastructure?

These and other questions remain for the future. So far, promising results have been obtained by applying two quantitative measures, frequency of beat and rate of dephosphorylation of ATP, to the isolated flagella. Hopefully it will be possible to measure other parameters and eventually to obtain a more nearly complete model of the rhythmic contractile process in flagella.

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Muscle Research and
Flagellar Movement

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All papers in this symposium have touched on the similarities in concept and experimental approach in the study of muscle and in the study of sperm. Bishop (this symposium) especially has enlarged on this theme, and has pointed out that, due to the technical difficulties in handling the material, the elucidation of the mechanism of movement in sperm has lagged behind the muscle studies. The natural consequence has been that the sperm physiologists have looked to the muscle literature for concepts and experimental breakthroughs which they then have applied to the study of the sperm cell with some success, and one now finds commonplace such terms as "contractile protein" and "relaxing factors" in the sperm literature.

There is an inherent danger in this approach, however, in that the sperm physiologists give too much credit to the muscle people; that is, they show a certain naiveté in accepting information from muscle studies. This results in a tendency of the sperm people to follow the lead of the muscle investigators too slavishly, with a consequent stifling of the possibility to develop new methods and approaches. It may therefore be well at this time to point out the inadequacies of the information from muscle, since the significance of this information has been well documented in this volume.

Molecular mechanisms of contraction are based largely on the wealth of information obtained from the study of so-called muscle models. This is a poor term, and the better term "simplified systems" is suggested, to include such inanimate systems as glycerinated muscle, extracted enzyme, and homogenates. These are all "simplified

muscle," if you wish. The point is that they are *not* living muscle, and the reactions and characteristics of a simplified system are not necessarily those which prevail *in vivo*. For example, there is widespread acceptance that muscle contraction is accompanied by the enzymatic splitting of ATP, and relaxation by the nonsplitting of ATP, a belief sustained by data from test-tube studies. But from living muscle studies there is no evidence to support this, and, in fact, the data of Chance (1959) and his collaborators demonstrate that in the course of a twitch not nearly enough ATP is split to account for the energy expressed by the muscle. I am not suggesting that the ATPase concept is incorrect, but only that it is not a proved *in vivo* fact, in spite of its wide acceptance.

The foregoing cautionary statement applies to all information from inanimate systems; thus, there is no proof of the existence of an *in vivo* relaxing factor, for example. There is, of course, a real value in studying simplified systems, and I do not mean to discourage this line of research but only to caution against the tendency to transfer without reservation results of such research to living muscle or living sperm.

We may move now from these general considerations to a more specific case where information from muscle research must be regarded with caution by the participants of this symposium, for the authors of the papers have enunciated two mechanisms of flagellar movement derived from muscle study. The first, as expressed by both Tibbs and Bishop at this symposium, calls for localized, propagated contractions and accompanying relaxations in the flagellar structure. The second, suggested by Brokaw (this symposium), involves active contraction of the flagellum coupled by feedback to internal viscosity and/or elasticity to give oscillatory movement. The first requires relaxation as a process, either the opposite of the contraction process or an entirely separate and new process which restores the contractile elements to their original state. The second does not need this; all that it requires is a cessation of the contractile process and one further condition, namely, that the contractile structures become more extensible after the cessation of the contraction process.

The question whether or not relaxation as a process exists in spermatozoa could resolve into whether or not a "relaxing factor" exists in flagella, and therefore merits discussion of the "relaxing factor"

information as obtained from muscle studies. It may be mentioned here that the discovery by Marsh (1952) of "relaxing factor" involved a counteraction by the factor of the ATP-induced volume change in muscle homogenates. In other words, this synaeresis was (and is) assumed to be equivalent to muscle contraction, and its counteraction equivalent to relaxation. Subsequently, other workers found that the factor caused a loss of tension (isometric) or an extension (isotonic) of ATP-contracted, glycerinated muscle cells, which brought its consideration much closer to that of relaxation in the living muscle.

The point that I would like to make is that, in the postulation of molecular mechanisms, the terms "relaxation" and "relaxing factor" are not precise enough. To make this clear, we may ask the simple question, "What sort of processes will bring about the end result of relaxation?" and it is easy to see that at least two kinds of processes, possibly more, can each produce what is called relaxation. These are (1) the reversal of a contractile process and (2) the "loosening" (plasticizing) of the contractile structure.

To use a crude analogy, picture a crank-and-pulley arrangement which pulls up a rope with a weight attached at the bottom end. The energetic upward cranking by which the weight is lifted is analogous to the contraction process. Cranking down, then, is the reversal of this process, and is analogous to relaxation. But suppose the bearing on the pulley loosens as the rope is being cranked up; the crank can continue to turn up, but the rope and weight drop. In other words, the effect of relaxation is achieved by two different mechanisms as given above. The question then arises, "Which of these two mechanisms does the 'relaxing factor' bring about?"

Experimental evidence to point up this dilemma can be cited. A single fiber of glycerinated psoas muscle can be isolated and mounted in a simple isotonic lever system. A load of about 100 mg can be imposed and ATP added to the surrounding bath, whereupon the fiber will contract and lift the load a distance equal to about 50% of the length of the fiber. Now "relaxing factor" is added to the bath, whereupon the fiber "relaxes," elongating to the original length. So far everything is as expected. However, if now half of the load is removed, reducing the load on the fiber to 50 mg, the fiber promptly contracts again! It is quite clear, therefore, that under conditions where "relaxation" occurs with a heavier load, the contractile proc-

ess is still going on (T. Hayashi and H. C. Lamont, unpublished results).

It is quite possible to interpret these results by mechanisms other than the alternatives stated above, but they will reduce, I believe, to complicated combinations of these alternatives, and necessitate added assumptions. The question is, "Is the 'relaxing factor' acting as a 'structure loosener' (plasticizer) or as a 'contractile-process reverser' (which may involve ATPase inhibition) in bringing about the relaxation phenomenon?"

All this is not to be taken as discrediting the work on "simplified systems." I am saying only that data are data and must be scrutinized for their limitations. With this in mind, we may now examine the question of contractile proteins in flagella.

It is interesting that a myosin-like protein has been found in sperm flagella by the Russian workers (see Bishop, this symposium). But work with muscle proteins has shown that myosin and actin together are an absolute requirement for contraction (Hayashi *et al.*, 1958). If we assume that the same prevails in sperm-tail proteins, then it behooves one to look for an actin-like protein. This may not be too difficult, for methods for extracting actin from muscle have been worked out quite completely (Ulbrecht *et al.*, 1960).

A very strong indication of the presence of actin may be obtained even more easily. Actin is characterized by having a nucleotide (either ATP or ADP) bound to it (Straub and Feuer, 1950), and this fact can be utilized analytically. An extract can be cleared of its contaminating, unbound nucleotides (Tsuboi and Hayashi, 1959), and once this is done, the bound nucleotide can be determined. If any exists, a strong presumption of the presence of actin would be indicated, and therefore the presence of a complete contractile system in the sperm tail. This information would be of importance, and I suggest that the "spermiologists" use this technique to establish it.

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Epilogue

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The degree of success of this symposium during which the foregoing papers were presented is evident from the contributions themselves. As an assemblage of surveys of recent findings and reports of new data, they indicate what has been accomplished and what we would like to know—in short, where we stand. In an evaluation, rather than a summary, of results of such a symposium, it behooves us to recall that a symposium, while literally a "drinking party," is also a "collection of opinions" on a given subject. The collection has been varied and necessarily tentative. It is certainly not the last word, as is painfully evident to one who completed, just prior to these meetings, a review of the subject which is already, in some respects, obviously obsolete (Bishop, 1962). Both current advances and certain major unresolved questions have been brought sharply into focus.

The reports from the University of Cambridge contribute significantly, as usual, to the understanding of wave formation and the nature of propulsion, while at the same time urging caution in interpretation and formulating a plea for technical innovation and improved observational methods. In the light of the closeness of fit of observed and calculated data of such parameters of sperm motility as velocity and power requirement, one accepts with tongue in cheek their warnings against their own overspeculation! Lord Rothschild's suggestion that calculations of biophysical data on a per sperm basis be taken not too seriously has some merit since, as he points out, we really do not know what the effect is of one sperm on an adjacent one. Indeed, Rikmenspoel's findings are significant in that they demonstrate an antagonistic repulsion of neighboring cells rather than a degree of thermodynamic cooperation extrapolated from the derivations of Sir Geoffrey Taylor. Nevertheless, in dealing with a population of cells, particularly one as clean and homogeneous as sperm

suspensions can be, there seems to be little harm, and perhaps some advantage, in expressing data in this fashion—so long as conditions are specified—rather than in terms of cell number, protein, nitrogen, wet or dry weights.

Rothschild's discussion of the form of the propagated wave suggests two commentaries. The nonsinusoidal shape of the planar wave as it appears in his slowed-down bull sperm preparation, if taken to mean a difference in amplitude on the two sides of the flagellum, augments other evidence suggesting that the beat is stronger on one side than the other. Assuming the direction of predominant bending bears a constant relation to internal and external structures of the sperm, we are led to the electron micrographic evidence presented by Fawcett for the bat sperm which indicates that a two-dimensional beat in the flagellum, like that of cilia, may be correlated with the position of the individual axial filaments. A more nearly complete picture has been revealed in slowed squid sperm (Bishop, 1958) in which the observed major planar wave corresponds to the position of the prominent spur of the midpiece, which in turn can be seen in electron micrographs to bear a constant relation to the individual axial filaments (Afzelius, personal communication). Another point noted by Rothschild is the variation in amplitude of the propagated wave as it passes down the flagellum of the slowed bull sperm. Wave amplitude normally increases distally in both bull and sea urchin sperm, whereas wavelength decreases in the former and increases in the latter (Gray, 1955; Rikmenspoel, 1957). The question arises whether these variations are to be attributed solely to the physical nature of the tensile and compressible elements of the sperm, or whether, in part at least, they represent differences in the energy supply locally available to the contractile system. Nelson comments on the fact that in rat epididymal sperm the proximal portion, anterior to the kinoplasmic droplet, can be quiescent while the distal segment undergoes active movement. Comparable wave distributions have been described in living squid sperm and in reactivated sperm model preparations.

Steinbach and Dunham have taken up a major task and seem to be making considerable, if iconoclastic, headway on a controversial subject—the nature of the permeability of the sperm membranes. It comes as a surprise to learn that the invertebrate sperm tail behaves,

ionically, as though no semipermeable membrane existed, but at least this may tend to clarify somewhat the doubt expressed by Rothschild and others that, under certain circumstances, ATP can penetrate and effectively activate the flagellum. If it is true, as sug- gested by Steinbach and Dunham, that the invertebrate "tails act like so many highly permeable strands of jelly," this could account for the absence of a glycolytic system in these flagella, in contrast to that found in mammalian sperm which are enclosed by a fibrous sheath and, presumably, a physiological membrane as well. It is to be hoped that this approach will be applied to an investigation of ionic balance in mammalian sperm and thereby elucidate the strange situations found there in regard to ionic tolerances (Bishop, 1962).

Many new ultrastructural details have been visualized in the elegant electron micrographs presented here by Fawcett and in the cyto- chemical localizations demonstrated by Nelson. One is made aware of an increasing structural complexity and optical differentiation of elements within the axial bundle. Additional fibrils and cross con- nections are indicated in the matrix. Although the standard $9 + 2$ axial fibrillar pattern is universal throughout flagellar and ciliary systems, the more restricted (mammals, birds, and possibly some in- vertebrates), outermost peripheral array of extra fibers has here re- ceived the most attention. Fawcett's interpretation of material from a number of mammalian species credits these electron-dense fibers with a major role in contractile movement, a view supported by Nelson's localization of antimyosin antibodies and ATPase activity in the same sites. One wonders whether similar methods of analysis can be applied to the simplified and more generally distributed type of motile organelle and, if so, where the myosin-like substance might be. In the $9 + 2$ cilia of the rotifer, Philodina, Lansing and Lamy (1961) find evidence, by a different technique, of ATPase in two areas outside the circular array of fibrils associated with filaments 1 and 5.

The abundance of cytochrome oxidase and succinic dehydrogenase in both the midpiece and *tail* of clam and bull sperm, alluded to by Nelson, presents a quandary in the light of the usual distribution of these enzymes and the general localization of the mitochondrial ap- paratus in the sperm midpiece. In some gametes, those of the honey- bee, for example, what appear to be mitochondria extend far down

into the principal segment of the tail (Rothschild, 1955). But in species like the sea urchin, clam, and mammals generally, there is little evidence that mitochondria are present outside the midpiece. It is not likely either that these two enzymes constitute part of an extramitochondrial respiratory system in the flagellum, particularly if the above-noted permeability data of Steinbach and Dunham find wide application (see also Gonse, this symposium). We feel rather confident that the energy for motility, in invertebrate sperm at least, comes from oxidative phosphorylation, and Rothschild and Cleland (1952) have demonstrated that endogenous phospholipid, located almost entirely or exclusively in the midpiece, serves as energy substrate. If for the moment just the invertebrate type is considered, we seem to be faced with the paradox of having a sperm with mitochondria and substrate limited to the midpiece while enzymes of the terminal electron transport chain, and presumably the sites of energy transfer as well, are distributed throughout the tail.

The simple pumping of ATP into a motile system does not explain how the mechanism works, but there is little basis for the argument that this nucleotide does not in some way facilitate the transfer of energy involved in chemomechanical coupling. Lord Rothschild's evidence that total nucleotide (ATP, ADP, and AMP + IMP) does not vary significantly during large changes in activity of anaerobic suspensions of bull sperm might be anticipated whether rephosphorylation did or did not occur. The details of these experiments should prove interesting. With respect to ATP levels alone, even in a nonphosphorylating system, it is a well-known fact that nucleotide changes are difficult to demonstrate with active muscle preparations (see also Hayashi, this symposium). In this connection it is worth calling attention to the conflicting evidence regarding the occurrence of phosphagen and a transphosphorylating system in sperm. The most recent study of mammalian spermatozoa indicates their absence (White and Griffiths, 1958); in sea urchin gametes, however, a CP reserve system is claimed to be present (Yanagisawa, 1959). If one assumes these findings are confirmed, dare one suggest that the presence or absence of a phosphagen system in invertebrate or mammalian sperm might reflect the marked metabolic differences and their relative capacities for rapid rephosphorylation of ADP? In light of the conclusion suggested in the ensuing paragraph, a

phosphagen system would prove of greater advantage to an inverte-
brate sperm than to a mammalian germ cell.

When the wealth of metabolic information concerning glycolytic
processes in sperm is considered, it is particularly refreshing to have
the remarkable comparative survey of qualitative and quantitative
aspects of respiratory pigments presented here by Gonse. It is sur-
prising, moreover, to learn of the general similarity of the cyto-
chromes, pyridine nucleotides, and flavoproteins when compared in
sperm of the bull, dog, and clam, Spisula. Whereas the relative pro-
portions of cytochromes may differ significantly, total concentration
and turnover number appear about the same. All three species carry
equivalent quantities of cytochromes, i.e., approximately 2.5×10^6
molecules per sperm (see also Perry *et al.*, 1960). Cytochrome oxidase
content is relatively large in the sperm of the dog and bull. From re-
sults with DNP, specific respiratory inhibitors, Amytal and antimy-
cin A, and the less specific inhibitor, azide, it would seem that clam
sperm are much more sensitive to uncoupling than are mammalian
sperm. Oxidative phosphorylation, while yielding a greater amount
of energy available (as ATP) for motility, does so at a slower and less
accessible rate, owing to compartmentation according to Gonse, than
is the case with glycolysis. One may, on this basis, account for the
preferential utilization of glycolysis over oxidative respiration by
mammalian sperm under aerobic conditions in the presence of suit-
able substrate.

A new twist to the fate of degradation products of the Embden-
Meyerhof glycolytic scheme in sperm has been outlined by Terner.
A considerable portion of the pyruvate is regarded as being con-
verted, by dismutation, to lactate, acetyl CoA and carbon dioxide,
under both aerobic and anaerobic conditions. Confirmation of this
metabolic pathway in sperm would seem to be indicated before one
speculates just how general the process might be. Whether or not,
however, acetyl CoA is thus made available for synthesis in sperm,
Terner reports on the presence of strongly labeled diglycerides, and
less intensely labeled triglycerides and phosphatid, in bull sperm in-
cubated with C^{14}-glucose. Flipse also finds evidence of synthetic
activity, if this is what is is, in bull sperm, namely, the formation of
the dipeptide carnosine in cells exposed to C^{14}-histidine. It would be
interesting to know whether sperm homogenates or extracts can per-

form these synthetic activities, comparable to dinucleotide formation by phosphorylases extracted from human sperm as reported by Hakim (1959).

The specific roles and interplay of a number of factors which can affect sperm respiration, glycolysis, and motility, *in vivo*, have been recorded by Salisbury in the excellent survey of bull sperm under a variety of conditions. It is well to be reminded of the paradoxical roles of K^+, PO_4^{---}, and CO_2, essential in small amounts and inhibitory at high concentrations. Some of the historical indictment has been lessened, also, against Ca^{++} as a divalent cation in diluting media. From methods described here, coupled with those involving studies of ionic balance noted above, there should soon unfold a fairly complete account of the precise physiological control of metabolism and motility of sperm in such significant biological niches as the mammalian epididymis.

In view of Salisbury's allusion to the so-called metabolic regulator of Lardy and colleagues, it should be noted that many of their data can be interpreted in terms of metabolic control mechanisms applicable to other, more generalized, cells (Bishop, 1961). Uncoupling agents and the relative concentrations and availability of inorganic phosphate and ADP are significant factors. Salisbury's work points up the inhibitory role of excess P_i on respiration and phosphorylation, and Rothschild's findings suggest a low rate of phosphorylation in freshly ejaculated sperm under anaerobic conditions as manifested by a relatively high ADP to ATP ratio.

Discussions such as we have had here concerning energy conversion for motility, efficiency of substrate utilization, metabolic control of movement, and the like, always seem to skirt the issue—what price motility? Dr. Carlson has come up with some answers. Any conceptual model has its limitations, to be sure, and his is a simplified model. But important connotations are implied by the relation of energy procurement versus energy expenditure. Under what conditions is it to the sperm's advantage to move at all? Nature may have had this in mind when she designed the *modus operandi* of the hemipteran (*Notonecta*) sperm which remain immotile until activated by female secretions, and of herring (*Clupea*) sperm which normally become motile only in the vicinity of the egg micropyle (Pantel and de Sinéty, 1906; Yanagimachi, 1957). The stirring-rod hypothesis

of flagellation is not likely to endear us to those who still believe that sperm have to swim the distance from emitter to egg, but there actually is little direct evidence that active motility is required by sperm of most animals at times other than the precise moment of fertilization. While most of us do not envisage spermatozoa as free-ranging, voracious predators, the selection by Carlson of food collection as the limiting parameter to energy expenditure seems reasonable. Exogenous substrate, and even molecular oxygen, within the genital tract of either sex can conceivably function as an essential prerequisite for sperm survival and could, under conditions of short supply or limited diffusion, constitute the controlling factor in establishing the balance between energy expenditure by movement and energy conservation through quiescence. With respect to spermatozoa, Carlson's postulates would involve survival rather than growth, and the selective value of any mechanism concerning energy balance would not be inconsistent with the genetic and physiological variability which occurs within sperm populations (see Bishop, 1961).

The contributions of Nelson, Pautard, Tibbs, Brokaw, and Bishop have brought up to date the status of the contractile proteins which can be extracted from, or demonstrated in, flagella. Depending on the source material, method of extraction, conditions of assay, and biochemical properties, these products appear roughly comparable to actin, myosin, or actomyosin. Their physical characteristics where known, response to ATP, splitting activity, ionic requirements, interaction with muscle proteins, and sensitivity to inhibitors and chelating agents suggest close parallels with functional proteins from muscle. The final characterization and identification, however, of none of these components are as yet complete, and forthcoming investigations must decide how distinctive or how similar to muscle proteins these sperm constituents may be. With only rare exception is the precise site of contractile material in the flagellum indicated—that localized by means of the antibody-absorption technique, combined with electron micrography, as demonstrated by Nelson. Generally, little change occurs in fibrillar ultrastructure with extraction of contractile components.

A very significant finding is Pautard's claim for an oscillating gel, extractable from fish sperm by a modified Weber-Edsall procedure. Whether this proves to be a new type of "oscillating protein," char-

acteristic of flagellar mechanisms, as distinct from a system of contractile proteins typical of muscle, remains to be seen. If the existence and operation of such a single macromolecular entity is established, it would favor the concept of a monocomponent rather than a bicomponent system of contractile elements in sperm.

How the contractile elements are built into the longitudinal filaments, if indeed they are, is currently an open question. Pautard suggests that they may be relatively short segments, not necessarily extending the length of the fibril, an idea also raised earlier by Gray. This problem might be further explored, along the lines suggested by Sir James and Lord Rothschild, by isolation of the fibrils in a functional state. The relative ease with which fish and fowl sperm, for example, can be longitudinally dissociated should not make this technically unfeasible.

Many other unresolved problems confront us. A basic question, one upon which the opinion of the symposium participants is split right down the middle, concerns the nature of the normal wave pattern, whether planar or helical. Some discrepancies may be attributed perhaps to species differences and others to methods of observations and recording of movement. Possibly what is needed is the construction of a wide-angle binocular microscopic device which can simultaneously view the same sperm at an angle of some 90 degrees, although even here one can expect some variation in interpretation of the actual waveform.

The tantalizing question of why $9 + 2$ fibrils as a universal pattern in flagella and cilia has arisen on numerous occasions and has been discussed with sound judgment by Rothschild. His point that the number 9 may be more closely associated with the intrinsic properties of centrioles of the division apparatus than with the requirements of the axial filaments which arise from a modified centriole is consonant with the observations of de Harven and Bernhard (1956) on the centrioles of a variety of vertebrate cells. The question would seem to be open to further attack by developmental studies of the axial bundle: its origin in the spermatid centriolar derivative and its differentiation in a sperm like that of Polyphemus, a crustacean, in which an amoeboid cell is claimed to transform, under experimental conditions, into a ciliated gamete (Geddes and Thomson, 1890).

The extent and nature of processes involving coordination within spermatozoa have been rather neglected, owing largely, one suspects, to the small size of the cells and the attendant technical difficulties. This area of sperm investigation, however, would seem to warrant extensive study. The commutator type of coordinating mechanism for wave initiation, as postulated by Bradfield (1955) for example, was probably erroneous, and there is little agreement on what might account for wave propagation and coordination along the sperm flagellum. Perhaps, as Brokaw notes for algal flagella, an extra coordinating mechanism is not required; the sensitivity to elongation of the contractile elements themselves might account for wave propagation along the tail. It does seem, however, that in mammalian sperm models, the capacity for coordination, along with wave propagation, is lost completely, whereas contractility is retained (Bishop, this symposium).

The primary wave motion of normal fresh sperm is generally, if not always, from the base of the flagellum toward the tip. Some mechanism would appear to initiate this beat (whether it be two- or three-dimensional), assuming that there is not a countermechanism to inhibit the initiation of wave propagation elsewhere along the tail. One might therefore expect to find periodic fluctuations of some kind at the base of the head or in the midpiece, which periodicity would correspond to flagellar rate: pulsed action potentials, cyclic metabolic changes, fluctuations in ionic balance or other periodic variations, however rapid and however small.

Just what role acetylcholine plays in sperm movement is quite obscure at present, but the occurrence of this chemical transmitter has been verified by both Tibbs and Nelson. Its unequivocal identification and localization in sperm in general would suggest that a transmissible or coordinating mechanism is at work.

The ultimate question—the nature of the mechanism of sperm motility—remains at the end of this symposium, as at the beginning, a major problem, and perhaps will continue so until another meeting a decade hence. Much of the reasoning and most of the speculation may be traced back to comparable processes and principles pertinent to muscle contraction and implicate folding protein chains, sliding filaments, oscillating fibrils, contractile enzymes, relaxing factors,

and the rest. Some or all of these features may apply to sperm flagellation but at present we simply do not know where to put them, much less operate them. Rather than close on the admonitory words of Hayashi who rapped the spermatological knuckles for overly faithful adherence to procedures and interpretations of investigators of muscle, one would hope that new methods and fresh approaches will be brought to bear on these many unresolved questions, perhaps unique to sperm physiology.

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Index